

2017

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Robinson, Bonnie L.; Dumas, Melanie; Ali, Syed F.; Paule, Merle G.; Gu, Qiang; and Kanungo, Jyotshna, "Cyclosporine exacerbates ketamine toxicity in zebrafish: Mechanistic studies on drug–drug interaction" (2017). *Food and Drug Administration Papers*. 4.
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Cyclosporine exacerbates ketamine toxicity in zebrafish: Mechanistic studies on drug–drug interaction

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ABSTRACT: Cyclosporine A (CsA) is an immunosuppressive drug commonly used in organ transplant patients to prevent allograft rejections. Ketamine is a pediatric anesthetic that noncompetitively inhibits the calcium-permeable *N*-methyl-D-aspartic acid receptors. Adverse drug–drug interaction effects between ketamine and CsA have been reported in mammals and humans. However, the mechanism of such drug–drug interaction is unclear. We have previously reported adverse effects of combination drugs, such as verapamil/ketamine and shown the mechanism through intervention by other drugs in zebrafish embryos. Here, we show that ketamine and CsA in combination produce developmental toxicity even leading to lethality in zebrafish larvae when exposure began at 24 h post-fertilization (hpf), whereas CsA did not cause any toxicity on its own. We also demonstrate that acetyl L-carnitine (ALCAR) completely reversed the adverse effects. Both ketamine and CsA are CYP3A4 substrates. Although ketamine and CsA independently altered the expression of the hepatic marker *CYP3A65*, a zebrafish ortholog of human *CYP3A4*, both drugs together induced further increase in *CYP3A65* expression. In the presence of ALCAR, however, *CYP3A65* expression was normalized. ALCAR has been shown to prevent ketamine toxicity in mammal and zebrafish. In conclusion, CsA exacerbated ketamine toxicity and ALCAR reversed the effects. These results, providing evidence for the first time on the reversal of the adverse effects of CsA/ketamine interaction by ALCAR, would prove useful in addressing potential occurrences of such toxicities in humans. Published 2017. This article is a U.S. Government work and is in the public domain in the USA.

Keywords: ketamine; zebrafish; acetyl L-carnitine; cyclosporine; CYP3A65

Introduction

Cyclosporine A (CsA), a neutral hydrophobic cyclic peptide of 11 amino acids, is a potent immunosuppressant used in organ transplantation patients and for treating various autoimmune diseases (Conde *et al.*, 2008). The use of CsA, however, has been associated with a number of side effects that include neurotoxicity (Chang *et al.*, 2001), hepatotoxicity (Galan *et al.*, 1995), nephrotoxicity (Woolfson and Neild, 1997) and hypertension (Textor *et al.*, 1994). Interaction of CsA with other drugs has been shown to cause altered blood levels of the drugs, a decrease or increase in effectiveness and side effects of both CsA and the co-administrated drugs (Backman *et al.*, 2006; Neuvonen *et al.*, 2006). Ketamine, a pediatric anesthetic, is an antagonist of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (Kohrs and Durieux, 1998). Ketamine is listed as a schedule III controlled substance in the USA, due to its potential for abuse (Morgan *et al.*, 2010; Rowland, 2005). It is also widely used in veterinary medicine to induce anesthesia and relieve postoperative pain (Wright, 1982). In many animal species including horses, ketamine is administered in combination with xylazine, diazepam and other compounds to avoid adverse effects associated with ketamine (Hazra *et al.*, 2008; Sinclair and Valverde, 2009). Although, xylazine/ketamine administration is considered safe for anesthesia in rats (Saranteas *et al.*, 2005), high mortality in CsA-treated rats when anesthetized with a common intraperitoneal dose of xylazine (10 mg kg⁻¹ body weight) and ketamine (100 mg kg⁻¹ body weight) has been reported (Loeffelbein *et al.*, 2010). Furthermore, in rats, a reduced ketamine dose, but not the xylazine

dose resulted in a significant increase in survival rate indicating that CsA and ketamine interaction are likely to occur (Loeffelbein *et al.*, 2010). Therefore, the need to reduce the anesthetic dose of ketamine in CsA-treated rats by about 40% has been recommended (Loeffelbein *et al.*, 2010).

Intraperitoneal application of xylazine/ketamine for anesthesia in rats co-administered with CsA caused high mortality possibly due to respiratory arrest brought upon by a relative overdose potentially resulting from altered cytochrome P (CYP)450 enzyme expression by CsA (Sato *et al.*, 2007). Ketamine is a substrate of CYP3A4 in rats (Meneguz *et al.*, 1999). It was therefore assumed that the dose reduction for ketamine in CsA-pretreated rats might be related to slower drug metabolism (Loeffelbein *et al.*, 2010). Marked variation in response to drugs among individuals is suggested to be associated with differences in CYP3A expression (Dresser *et al.*, 2000). When pre-treated with other drugs such as chloramphenicol, cimetidine, ketoconazole or SKF 525-A (substrates of CYP3A), prolonged anesthesia and increased mortality after xylazine/ketamine administration in rats were reported (Amouzadeh *et al.*, 1989). The interaction of CsA with barbiturates (anesthetic drugs) in mice also induced prolonged

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sleeping time (Sato *et al.*, 2007). Furthermore, ketamine in a patient immunosuppressed with CsA has been shown to be unsafe as both the drugs with possible proconvulsant properties induced generalized tonic clonic seizures (Agarwal *et al.*, 2005).

The immunosuppressant property of CsA is based on its ability to inhibit calcineurin signaling (Yu *et al.*, 2013). Although CsA is the first choice of drug during organ transplantation, it has been shown to cause serious nephrotoxicity, hepatotoxicity, neurotoxicity and cardiotoxicity (Rezzani, 2004). Drug–drug interactions pose serious issues for human health. Many drug–drug interactions can result due to alterations in their metabolic enzymes (e.g., CYPs), which are mostly expressed in the liver and gut. During co-administration, while some drugs act as inducers of the enzyme(s), others act as inhibitors. Zebrafish embryos and larvae in recent years have been used as alternative *in vivo* toxicity screening models for teratogenesis as well as organ-specific toxicities in the early drug discovery process (Eimon and Rubinstein, 2009; Kanungo *et al.*, 2014; McGrath and Li, 2008). Zebrafish larvae have a fully functional liver at 72 hpf and they express 94 CYP enzymes, most being human orthologs (Goldstone *et al.*, 2010). As in humans, zebrafish CYP1–4 enzymes metabolize xenobiotics, while the zebrafish CYP3A65 has been identified as the ortholog of the human CYP3A4 (Goldstone *et al.*, 2010; Hill *et al.*, 2012; McGrath and Li, 2008). Similar to human CYP3A, enhanced CYP3A65 transcription in the foregut of the zebrafish larvae in response to dexamethasone and the antibiotic rifampicin confirmed that zebrafish CYP3A65 is an ortholog of the human CYP3A gene (Chang *et al.*, 2013; Tseng *et al.*, 2005). CYP3A65 expression in zebrafish has been used as a hepatic biomarker (Al-Habsi *et al.*, 2016; Cunha *et al.*, 2016; Verstraelen *et al.*, 2016; Xia *et al.*, 2016) and the zebrafish embryo model was shown to detect hepatotoxics with higher specificity than the HepG2 cells (Jones *et al.*, 2009).

The objective of the present study was to assess CsA/ketamine drug–drug interaction effects on the zebrafish larvae and to elucidate potential mechanisms by reversing the adverse effects with a third drug. Additionally, evaluation of the CYP3A65 (zebrafish ortholog of the human CYP3A4) expression was undertaken to determine possible link to the drug interaction effects.

Materials and methods

Animals

Adult wild-type zebrafish (*Danio rerio*, AB strain) were obtained from the Zebrafish International Resource Center (www.zirc.org) (Eugene, OR, USA). The fish were kept in fish tanks (Aquatic Habitats, FL, USA) at the NCTR/FDA zebrafish facility containing buffered water (pH 7.5) at 28°C, and were fed daily live brine shrimp and Zeigler dried flake food (Zeiglers, Gardeners, PA, USA). Each 3 liter tank housed eight adult males or females. Handling and maintenance of zebrafish complied with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the NCTR/FDA IACUC. The day/night cycle was maintained at 14/10 h. For in-system breeding, crosses of males and females were set up the previous day with partitions that were taken off the following morning at the time of light onset at 07.30 h to stimulate spawning and fertilization. Fertilized eggs were collected from the bottom of the tank as soon as they were laid. The eggs were placed in Petri dishes and washed thoroughly with buffered egg water (reverse osmosis water containing 60 mg

sea salt [Crystal Sea®, Aquatic Eco-systems, Inc., Apopka, FL, USA] per liter of water, pH 7.5) and then allowed to develop in an incubator at 28.5°C for later use.

Reagents

Ketamine hydrochloride was purchased from Vedco, Inc. (St. Joseph, MO, USA). Acetyl L-carnitine (ALCAR) and CsA were purchased from Sigma (St. Louis, MO, USA). ALCAR stock (1 M) solutions were made fresh with buffered egg water. CsA stock (10 mM) was prepared using dimethyl sulfoxide (DMSO) as the solvent. All other reagents used in this study were purchased from Sigma unless mentioned otherwise.

Treatment of zebrafish embryos with ketamine, cyclosporine a and acetyl L-carnitine

As we previously reported, ALCAR at 0.5 mM did not have any effect on the zebrafish embryo development when exposed for 20–24 h, and 0.1 mM ALCAR did not prevent ketamine-toxicity. However, doses of 0.5 or 1.0 mM was effective in completely inhibiting ketamine's adverse effects (Cuevas *et al.*, 2013; Guo *et al.*, 2017; Kanungo *et al.*, 2012). Based on these data we chose to use 1.0 mM ALCAR for all our experiments in the present study. For ketamine dose, we have earlier determined that 2 mM ketamine in water results in an internal exposure of the anesthetic level of plasma concentrations in humans (Trickler *et al.*, 2014). For co-exposure to various drug combinations, the drugs were added together at the same time. For the first experiment aimed at elucidating ketamine and CsA interaction effects, treatment with ketamine (2 mM), CsA (10 µM) and ALCAR (1.0 mM) was undertaken using manually dechorionated 24 h postfertilization (hpf) embryos. The specified doses of ketamine and ALCAR were used based on our multiple earlier studies (Cuevas *et al.*, 2013; Kanungo *et al.*, 2012, 2013; Lantz-McPeak *et al.*, 2015; Trickler *et al.*, 2014). We used 10 µM CsA to elicit maximal effect of ketamine and CsA combination effects so as to determine whether ALCAR was effective against the drastic adverse effects. For each treatment 10 embryos were placed in each well of the six-well plates containing 5 ml buffered egg water (reverse osmosis water containing 60 mg sea salt [Crystal Sea®, Aquatic Eco-systems, Inc., Apopka, FL, USA] per liter of water, pH 7.5). Five replicates for each group were set up with a total of 50 embryos for each treatment group. The control group received 5 µl of DMSO (vehicle) with a final concentration of (0.1%). The experiment was repeated three times. Static exposure continued for 72 h. Beginning 72 hpf, zebrafish embryos are called larvae instead of embryos. The effects of the drugs were assessed in the larvae at 96 hpf. Survival of the larvae was scored using all five replicates ($n=50$). Body length was measured for all surviving larvae in the 2 mM ketamine + 10 µM CsA group (of 50). For other groups, body length was measured for two larvae from each well ($n=10$). Images of the embryos and larvae were acquired using a DP2 BSW microscope (Olympus, Tokyo, Japan). Body length was quantitated using a DP2 BSW microscope digital camera software (Olympus).

In the second set of experiments, 6 hpf embryos (with chorions intact) were used. The embryos ($n=10$ per well of a six-well plate in 5 ml buffered egg water) were exposed for 24 h (actual age of endpoint assessment is 30 hpf), 48 h (actual age of endpoint assessment is 54 hpf) and 72 h (actual age of endpoint assessments is 78 hpf) with 5 µl DMSO, 2 mM ketamine, 10 mM CsA, 2 mM ketamine + 10 µM CsA, 2 mM ketamine + 1.0 mM ALCAR,

2 mM ketamine + 10 μ M CsA + 1.0 mM ALCAR. The experiment was repeated three times. Images of the embryos and larvae were acquired using a DP2 BSW microscope. Body length was quantitated ($n=10$) using a DP2 BSW microscope digital camera software.

For the third set of experiments aimed at mechanistic studies, a shorter exposure (24 h) of 72 hpf larvae was designed to avoid deformity and mortality. The 72 hpf larvae have guts and livers (Wilkins and Pack, 2013) that express drug-metabolizing CYP enzymes (Chang *et al.*, 2013). In a six-well plate, each well containing 10 larvae (72 hpf), exposure to 2 mM ketamine in the presence or absence of various sublethal doses of CsA (0.5, 1.0, 2.0 and 5.0 μ M) was performed. Another group received ALCAR (1.0 mM) in addition to 2 mM ketamine and 5.0 μ M CsA. Untreated control groups (exposed to equivalent amount of the vehicle, DMSO) were examined in parallel. Images of the embryos and larvae were acquired using a DP2 BSW microscope. Body length was quantitated ($n=10$) using a DP2 BSW microscope digital camera software.

The statistical significance of the effects of the various treatments on survival and body length was determined by one-way ANOVA (Sigma Stat) using Holm–Sidak pairwise multiple comparison *post-hoc* analysis. Statistical significance was based on $P < 0.05$.

RNA extraction and cDNA synthesis

Total RNA (from 50 pooled larvae per treatment group) was extracted from whole larvae using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). An aliquot of each RNA sample was used to determine spectrophotometrically (using a NanoDrop ND-1000; NanoDrop Technology, Wilmington, DE, USA) the RNA quality ($A_{260}/A_{280} > 2.0$) and concentration. First-strand cDNA was synthesized from total RNA (2 μ g; 20 ml final reaction volume) with oligo(dT) priming using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Primers

Zebrafish gene-specific primers (Table 1) were used for the real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays to quantify *GAPDH* and *CYP3A65* (GenBank entry no. NM_001037438.1).

Real-time quantitative reverse transcription polymerase chain reaction assay for gene expression analysis

Real-time qPCR was performed using a CFX96 C1000 (Bio-Rad, Hercules, CA, USA) detection system with SYBR green fluorescent label (Bio-Rad). Samples (25 μ l final volume) contained the following: 1 \times SYBR green master mix (Bio-Rad), 5 pmol of each primer and 0.25 μ l of the reverse transcription reaction mixture. Samples were run in triplicate in optically clear 96-well plates. Cycling parameters were as follows: 50°C 2 min, 95°C 10 min,

then 40 cycles of 95°C 15 s, 60°C 1 min. A melting temperature-determining dissociation step was performed at 95°C 15 s, 60°C 15 s and 95°C 15 s at the end of the amplification phase. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative gene expression (Livak & Schmittgen, 2001). The *GAPDH* gene was the internal control for all qPCR experiments. Data from each group ($n=3$) were averaged and shown as normalized gene expression with SD. One-way ANOVA (Sigma-Stat) and Holm–Sidak pairwise multiple comparison *post-hoc* analyses were used to determine statistical significance. Statistical significance was based on $P < 0.05$.

Results

Acetyl L-carnitine protects zebrafish embryos/larvae from toxicities induced by ketamine and cyclosporine a

Manually dechorionated 24 hpf zebrafish embryos were treated with either 2 mM ketamine or 10 μ M CsA for 72 h. Compared to control, at 96 hpf, 2 mM ketamine induced developmental toxicity (Fig. 1A) with no mortality (Fig. 1B). The observed developmental toxicity included an edematous pericardium and significantly reduced linear body length (Fig. 1A,C). CsA on the other hand did not have any effects on either the pericardium (Fig. 1A) or body length (Fig. 1C). However, 2 mM ketamine in the presence of 10 μ M CsA induced severe deformity in the larvae (Fig. 1A) and 80% mortality (Fig. 1B). The moderate toxicity induced by ketamine and severe toxicity on the overall development and survival induced by ketamine and CsA together were prevented with 1.0 mM ALCAR (Fig. 1A–C).

When treatment started at 6 hpf, developmental trends were similar to what occurred when 24 hpf embryos were treated. When compared to control and ALCAR co-treated embryos, embryos with 24 h of exposure (actual age 30 hpf) to ketamine and ketamine/CsA showed retarded overall development with less pigmentation and smaller eyes being the most obvious indicators (Fig. 2, upper panel). Similar trends were also observed when exposure continued up to 48 h (actual age of the embryos was 54 hpf) (Fig. 2, lower panel). At 72 h of exposure, larvae (actual age 78 hpf) in the control and ALCAR co-treated groups were all hatched and alive (Fig. 3A,C,E,F), although the ones exposed to the drugs had significantly reduced body length (Fig. 3G). On the other hand, larvae treated with ketamine or ketamine/CsA were unhatched and severely malformed (Fig. 3B,D). Although ALCAR prevented morphological deformity and mortality of the larvae treated with ketamine and ketamine/CsA, compared to control (Fig. 3A), these larvae had significantly reduced body length (Fig. 3–E,F). Even larvae treated with only CsA (10 μ M) also exhibited significantly shorter body length (Fig. 3C) than the control (Fig. 3A). These results indicate that very early exposure (6 hpf gastrula stage with intact chorions) results in incomplete reversal of ketamine and CsA toxicity by ALCAR.

Based on these data, we chose to focus our study using 24 hpf embryos or older for drug exposure as these embryos have all

Table 1. List of primers used in real-time quantitative polymerase chain reaction (RT-qPCR) assays

Gene	Forward primer	Reverse primer
GAPDH	5'-GATACACGGAGCACCAGGTT-3'	5'-GCCATCAGGTCACATACACG-3'
CYP3A65	5'-GAAACTGCAGGAGGATCG-3'	5'-CAAGTCTTTGGGGATGAGGA-3'

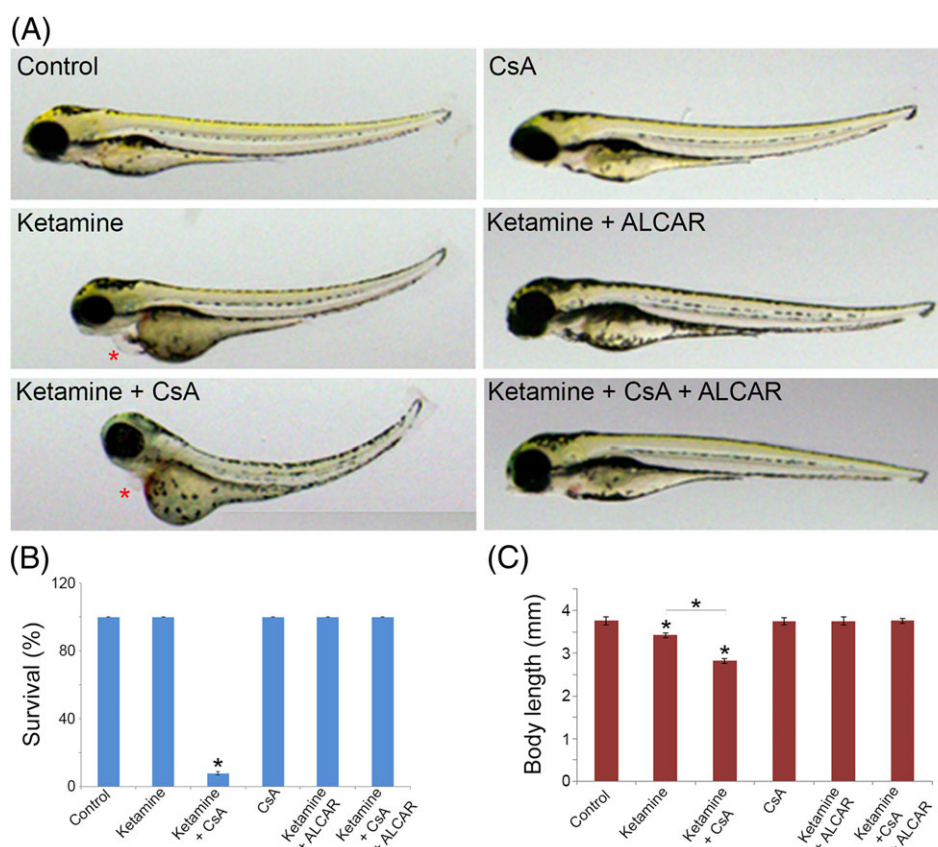


Figure 1. ALCAR protects zebrafish embryos from toxicities induced by ketamine and CsA in combination. (A) Zebrafish embryos at 24 hpf (manually dechorionated) were exposed (static exposure) for 72 h to: vehicle (dimethyl sulfoxide); 2 mM ketamine; 10 μ M CsA; 2 mM ketamine + 10 μ M CsA; 2 mM ketamine + 1.0 mM ALCAR; and 2 mM ketamine + 10 μ M CsA + 1.0 mM ALCAR. Images of 96 h larvae show severe pericardial edema (*). Ten embryos were treated for each group in each experiment. Control groups were treated with 5 μ l dimethyl sulfoxide (0.1%). The experiment was repeated five times. (B) Survival percentage ($n = 50$) of the larvae (96 hpf) as shown in (A). Data are presented as mean \pm SD. Statistical significance ($P < 0.05$) is indicated (*). (C) Linear body length ($n = 10$) was measured using the DP2 BSW microscope digital camera software (Olympus). Data are presented as mean \pm SD. Statistical significance ($P < 0.05$) is indicated (*). ALCAR, acetyl L-carnitine; CsA, cyclosporine A.

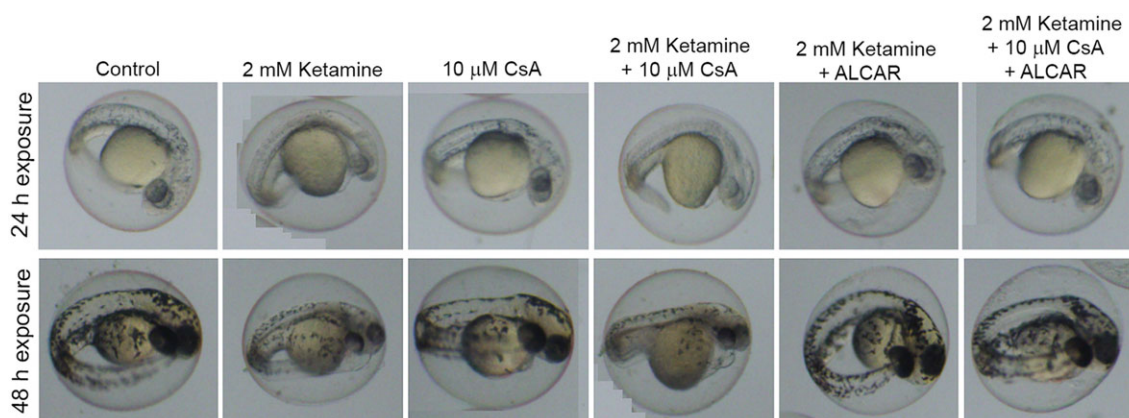


Figure 2. ALCAR protects zebrafish embryos from toxicities resulting from early exposure of embryos to ketamine and ketamine/CsA. Zebrafish embryos at 6 hpf (with intact chorion) were exposed (static exposure) for 24 h (upper panel) and 48 h (lower panel) to: vehicle (dimethyl sulfoxide); 2 mM ketamine; 10 μ M CsA; 2 mM ketamine + 10 μ M CsA; 2 mM ketamine + 1.0 mM ALCAR; and 2 mM ketamine + 10 μ M CsA + 1.0 mM ALCAR. Representative images of embryos (actual age, 30 hpf [upper panel] and 54 hpf [lower panel], respectively) show morphological features that indicate differences in body pigmentation and eye size. Ten embryos were treated for each group in each experiment. Control (vehicle) groups were treated with 5 μ l dimethyl sulfoxide (0.1%). Experiment was repeated three times. ALCAR, acetyl L-carnitine; CsA, cyclosporine A.

the organs or organ rudiments present and their response to the drugs would be more comparable to whole organisms. The results indicated that CsA exacerbated ketamine toxicity as CsA has no

adverse effects on its own on the zebrafish larvae. ALCAR's reversal of the CsA and ketamine combination toxicity also confirmed that it was ketamine toxicity that was intensified with CsA

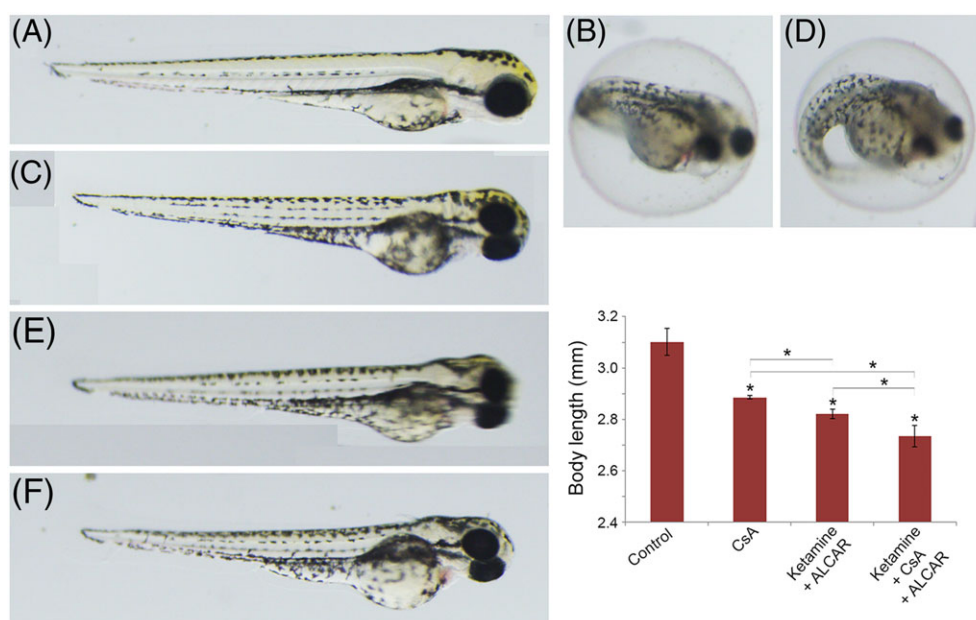


Figure 3. Early exposure of ketamine and ketamine/CsA in the absence of ALCAR causes extreme deformity in zebrafish embryos. Zebrafish embryos at 6 hpf (with intact chorion) were exposed (static exposure for 72 h) to: (A) vehicle (dimethyl sulfoxide); (B) 2 mM ketamine; (C) 10 μ M CsA; (D) 2 mM ketamine + 10 μ M CsA; (E) 2 mM ketamine + 1.0 mM ALCAR; and (F) 2 mM ketamine + 10 μ M CsA + 1.0 mM ALCAR. Representative images of embryos (actual age 78 hpf) show morphology. Ten embryos were treated for each group in each experiment. Control groups were treated with 5 μ l dimethyl sulfoxide (0.1%). Experiment was repeated three times. Linear body length ($n = 10$) was measured using the DP2 BSW microscope digital camera software (Olympus). Data are presented as mean \pm SD. Statistical significance ($P < 0.05$) is indicated (*). ALCAR, acetyl L-carnitine; CsA, cyclosporine A.

co-treatment, as our previous studies have established that ALCAR prevents ketamine toxicity (Cuevas *et al.*, 2013; Guo *et al.*, 2017; Kanungo *et al.*, 2012).

Cyclosporine a dose-dependently exacerbates ketamine toxicity

To determine whether CsA enhances ketamine toxicity in a dose-dependent manner, we used 72 hpf zebrafish larvae. The goal behind this experiment was to use larvae with livers and guts (absent in 24 hpf embryos) that express drug-metabolizing enzymes. We also used lower doses of CsA to avoid lethality and to quantitate subtle endpoints of overall developmental changes. The larvae were treated only for 24 h, once again ascertaining that the effects would be milder and endpoints quantifiable. Post-exposure, at 96 hpf, compared to control, 2 mM ketamine induced pericardial edema in the larvae (Fig. 4A). Co-exposure to 2 mM ketamine with increasing doses of CsA (0.5, 1.0, 2.0 and 5.0 μ M) produced significant reductions in body length (Fig. 4B) and all the dose groups developed pericardial edema (Fig. 4A). ALCAR (1.0 mM) co-treatment prevented these developmental defects from occurring (Fig. 4A) and the body lengths of the larvae were comparable to control (Fig. 4B). These results further confirmed that CsA exacerbates ketamine toxicity dose-dependently and ALCAR prevents it.

Ketamine and cyclosporine a induce CYP3A65 expression

CYP3A65 is the zebrafish ortholog of human CYP3A4. Ketamine and CsA are substrates of the mammalian and human CYP3A4 enzyme. We explored whether ketamine and CsA altered the expression of CYP3A65 at the transcriptional level. Zebrafish larvae at 72 hpf were exposed to various doses of ketamine or CsA for 24 h. Ketamine

dose-dependently (0.5, 2.0 and 5.0 mM) induced CYP3A65 expression (Fig. 5A). CsA at various doses (0.5, 1.0, 2.0 and 5.0 μ M) also induced CYP3A65 expression (Fig. 5B). When 72 hpf larvae were exposed for 24 h to 2 mM ketamine with increasing doses of CsA (0.5, 1.0, 2.0 and 5.0 μ M), the higher three doses (1.0, 2.0 and 5.0 μ M) significantly upregulated CYP3A65 expression (Fig. 6A). These results suggested that ketamine and CsA (1.0–5.0 μ M) together might alter their metabolism by modulating the expression of CYP3A65.

Acetyl L-carnitine normalizes CYP3A65 expression induced by ketamine and cyclosporine a

As ALCAR prevents ketamine toxicity as well as ketamine/CsA combination toxicity, we explored whether it also differentially affected CYP3A65 expression that is induced by both the drugs alone or together. RT-qPCR analyses showed that ALCAR normalized CYP3A65 expression in the presence of ketamine and CsA (Fig. 6B). These results indicated that ALCAR has the ability to prevent zebrafish larvae from potential hepatic dysfunction induced by ketamine and CsA.

Discussion

CsA has been used as an immunosuppressant in organ transplantation patients (Kahan, 1989) as well as for treating immune diseases (Faulds *et al.*, 1993) for more than three decades. However, CsA's efficacy is challenged by severe side effects, such as nephrotoxicity, arterial hypertension and cancer progression (Guada *et al.*, 2016). High mortality occurred in CsA-treated rats when anesthetized with ketamine (Loeffelbein *et al.*, 2010). Hence, a reduction in the anesthetic dose of ketamine in CsA-treated rats by about 40% to avoid this adverse effect was recommended

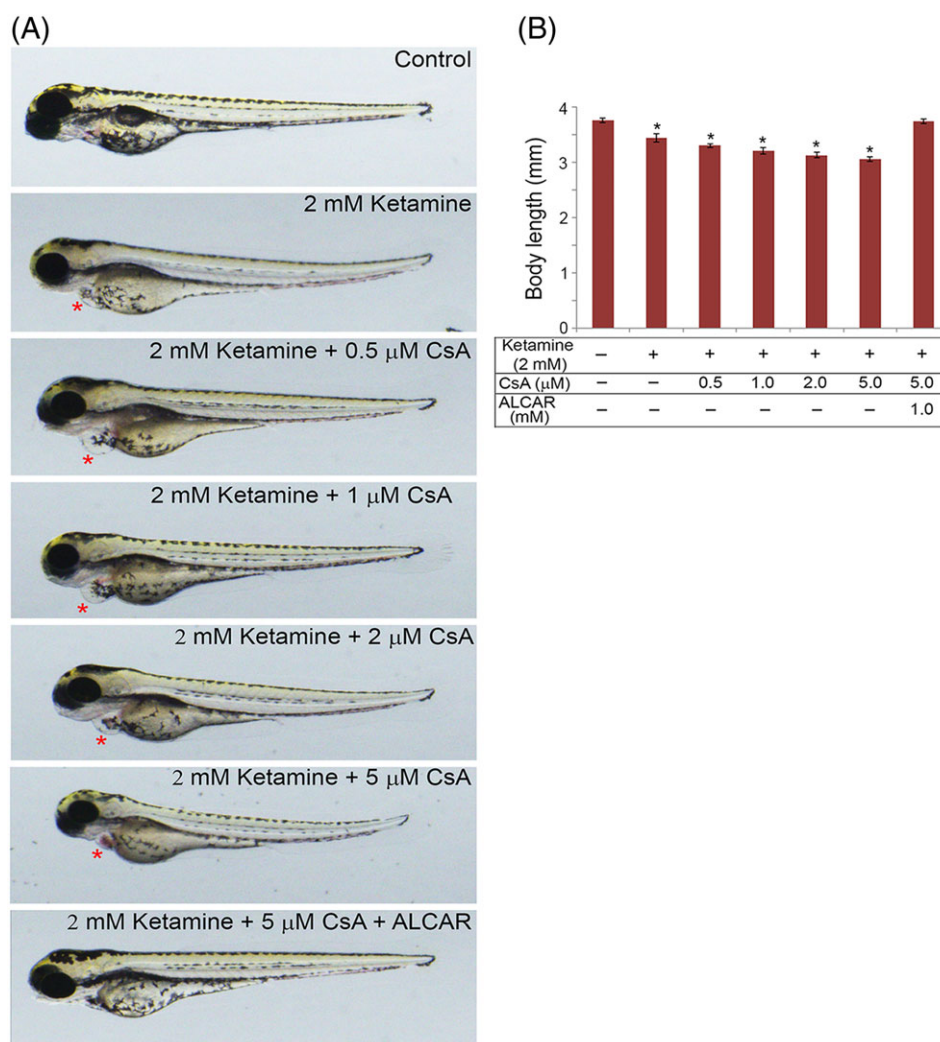


Figure 4. Dose-dependent effects of CsA on the zebrafish larvae in the presence of ketamine and ALCAR. (A) Zebrafish embryos at 72 hpf were exposed for 24 h to: 2 mM ketamine; 2 mM ketamine + 0.5 μ M CsA; 2 mM ketamine + 1.0 μ M CsA; 2 mM ketamine + 2 μ M CsA; 2 mM ketamine + 5 μ M CsA; and 2 mM ketamine + 5 μ M CsA + 1.0 mM ALCAR. Ten embryos were treated for each group in each experiment. Control groups were treated with 2.5 μ l dimethyl sulfoxide (0.05%). Experiment was repeated three times. Images (lateral views) of the 96 hpf larvae are shown. Pericardial edema is marked with an asterisk. (B) Body lengths of the larvae ($n = 10$) treated with ketamine, CsA and ALCAR as indicated above are presented as mean \pm SD. Statistical significance ($P < 0.05$) is indicated (*). ALCAR, acetyl L-carnitine; CsA, cyclosporine A.

(Loeffelbein *et al.*, 2010). Ketamine in a patient immunosuppressed with CsA has been shown to be unsafe as both the drugs induced generalized tonic clonic seizures (Agarwal *et al.*, 2005). Our results with 24 hpf embryos co-treated with 10 μ M CsA and 2 mM ketamine for 72 h produced adverse effects with about 90% mortality occurring in the 96 hpf larvae. The 10 μ M (12 mg l^{-1}) CsA concentration is higher than the 0.8–2 mg l^{-1} in the serum of patients who take CsA for immunosuppression (Keown, 2002); although the internal CsA concentration in the zebrafish larvae could presumably be much lower than 10 μ M added to the water as, in humans, bioabsorption of cyclosporine is significantly decreased than the actual dose in humans (Drewe *et al.*, 1992). Additionally, in zebrafish embryos, other drugs, such as ketamine and ethanol have been shown to have internal concentrations of only about 0.4% and 0.86%, respectively (Ali *et al.*, 2011; Trickler *et al.*, 2014). Ketamine and CsA co-treatment significantly reduced body length in the surviving larvae compared to the ketamine-treated larvae that showed no mortality despite being significantly smaller than control.

Mostly focusing on the overall development with a major objective to reverse the adverse effects, we observed pericardial edema in the larvae treated with ketamine alone. Surviving larvae of the CsA/ketamine co-treatment group also showed pericardial edema and a severely deformed body axis. As CsA-treated larvae developed normally as control, it is plausible that CsA exacerbated ketamine toxicity as reported in rats (Loeffelbein *et al.*, 2010), mice (Sato *et al.*, 2007) and human (Agarwal *et al.*, 2005). It is further confirmed by the reversal of the adverse effects by ALCAR as ALCAR has been shown to prevent ketamine toxicity in zebrafish (Cuevas *et al.*, 2013; Guo *et al.*, 2017; Kanungo *et al.*, 2012), rats (Boctor and Ferguson, 2009) and cultured rat cortical neurons *in vitro* (Zou *et al.*, 2008). The results of ketamine/CsA toxicity reversed by ALCAR may shed light on human conditions resulting from CsA and ketamine interaction (<http://www.ehealthme.com/drug-interaction/ketamine%20hydrochloride/cyclosporine/>).

In mammalian cells, ketamine induces a blockade of lipid oxidation and a decrease in ATP content in rat heart, whereas ALCAR facilitates fatty acid oxidation to support ATP production

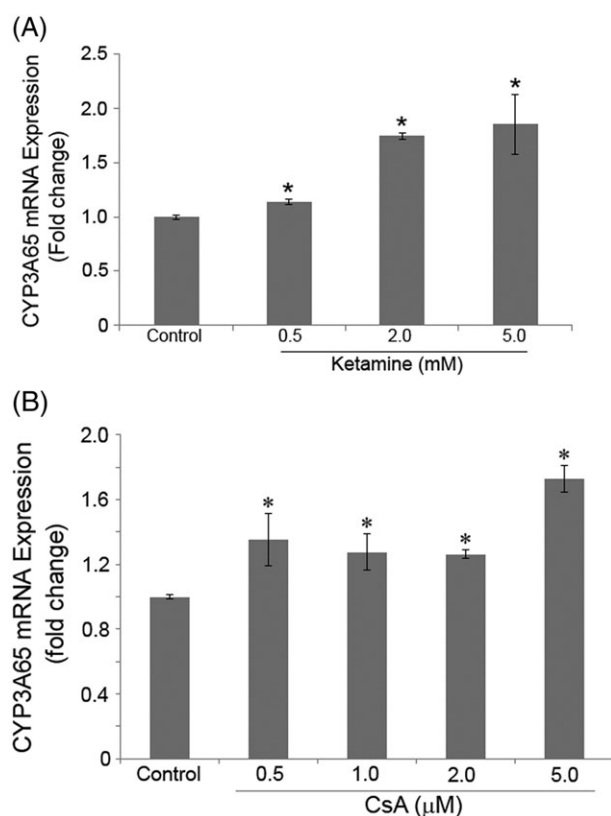


Figure 5. Ketamine and CsA alter the expression of CYP3A65 mRNA. (A) Zebrafish larvae at 72 hpf were exposed for 24 h with 0.5, 2 and 5 mM ketamine. Relative expression of CYP3A5 is presented (mean \pm SD) following qRT-PCR assessment of the cDNA synthesized from total RNA isolated from these larvae. Statistical significance ($P < 0.05$) is indicated (*). (B) Zebrafish larvae at 72 hpf were exposed for 24 h with 0.5, 1.0, 2 and 5 μ M CsA. Control groups were treated with 2.5 μ l dimethyl sulfoxide (0.05%). Relative expression of CYP3A5 is presented (mean \pm SD) following qRT-PCR assessment of the cDNA synthesized from total RNA isolated from these larvae. Statistical significance ($P < 0.05$) is indicated (*). CsA, cyclosporine A; qRT-PCR, reverse transcription–quantitative polymerase chain reaction.

(Hagen *et al.*, 2002). CsA produces hypoxia-like conditions in the liver (Zhong *et al.*, 2001), which causes depletion of mitochondrial energy (Garnier *et al.*, 1996; Murray and Paller, 1986). The reason CsA amplified ketamine toxicity in the zebrafish larvae could be attributed to ketamine's ability to reduce ATP production along with CsA's inhibition of cardiac high-energy phosphate and Krebs' cycle metabolism with decreased glutamate/glutamine concentrations and mitochondrial oxidative phosphorylation with reduction in ATP (Christians *et al.*, 2004). CsA showed a dose-dependent inhibition of oxidative phosphorylation and ATP production in rat kidney mitochondria (Nassberger, 1990) and inhibited cellular respiration with stimulation of ATPase activity in isolated mitochondria from mice (Giorgio *et al.*, 2010). These scenarios could explain the beneficial effects of ALCAR on ketamine-treated as well as ketamine/CsA co-treated larvae, which are further supported by our earlier finding that ALCAR's protective effects appear to be dependent on ATP synthase activity in zebrafish (Guo *et al.*, 2017).

Additional support on CsA's amplification of ketamine toxicity derives from our observation that increasing doses of CsA (0.5–5.0 μ M) proportionately inhibited overall development

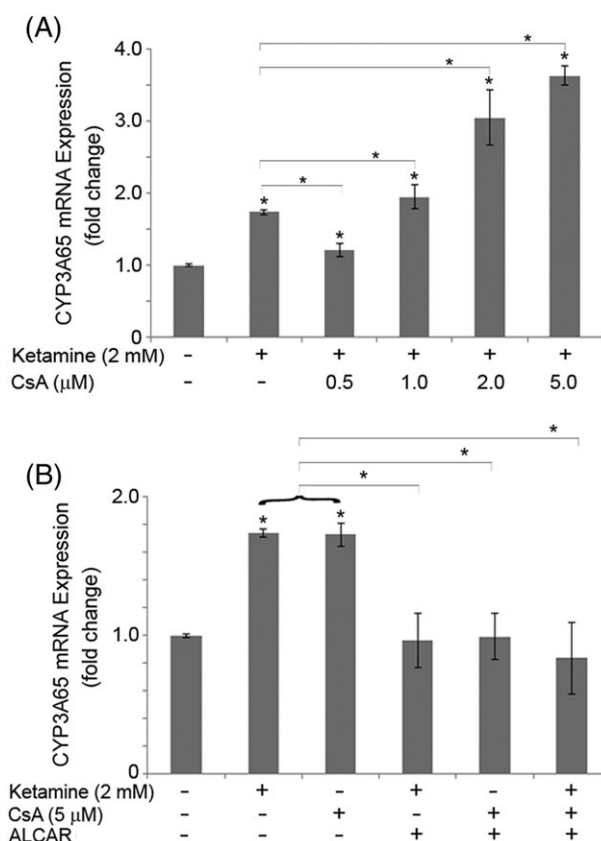


Figure 6. Ketamine and CsA upregulate CYP3A65 mRNA expression in the absence of ALCAR. (A) Zebrafish larvae at 72 hpf were exposed for 24 h with 2 mM ketamine alone or in the presence of 0.5, 1.0, 2 and 5 μ M CsA. Control groups were treated with 2.5 μ l dimethyl sulfoxide (0.05%). Relative expression of CYP3A5 is presented (mean \pm SD) following qRT-PCR assessment of the cDNA synthesized from total RNA isolated from these larvae. Statistical significance ($P < 0.05$) is indicated (*). (B) ALCAR normalizes CYP3A65 mRNA expression induced by ketamine and CsA. Zebrafish larvae at 72 hpf were exposed for 24 h with 2 mM ketamine in the presence or absence of CsA (5 μ M) and ALCAR (1.0 mM). Control groups were treated with 2.5 μ l dimethyl sulfoxide (0.05%). Relative expression of CYP3A5 is presented (mean \pm SD) following qRT-PCR assessment of the cDNA synthesized from total RNA isolated from these larvae. Statistical significance ($P < 0.05$) is indicated (*). ALCAR, acetyl L-carnitine; CsA, cyclosporine A; qRT-PCR, reverse transcription–quantitative polymerase chain reaction.

as reflected by body length in ketamine co-treated larvae. We have shown earlier that changes in body length serves as a reliable endpoint of developmental toxicity in zebrafish (Lantz-McPeak *et al.*, 2015). CsA disrupted Notch signaling resulting in vascular collapse in the zebrafish embryos when treatment started on freshly laid eggs that are at one-cell or early cleavage stages (Pandey *et al.*, 2015). It is not possible to compare these results with ours, as we exposed whole organisms (24 hpf) to CsA. Our study can therefore come close to recapitulating a holistic response to the drugs instead of a cellular response and is consistent with one other report that embryos treated with CsA (10 μ g ml^{-1}) from the one-cell stage (freshly laid egg) appeared morphologically wild type at 72 hpf (Beis *et al.*, 2005). However, when static exposure started at 6 hpf, we noticed developmental arrest that completely blocked hatching with severe pericardial edema at 78 hpf (72 h exposure) in the larvae treated with ketamine/CsA or ketamine alone, all of which died the same day a few hours apart, although

the ones treated with only 10 μM CsA were alive. The difference in the tolerance to the effects of ketamine and ketamine/CsA treatment of the 24 hpf embryos compared to the 6 hpf gastrulae indicates that a whole organism responds to drugs differently (possibly more specifically) from the developing gastrulae (6 hpf) that have not developed any organ rudiments but are an assembly of cells belonging to the embryonic germ layers (Keller *et al.*, 2008). Therefore, to test for toxicity of drugs, we prefer using 24 hpf zebrafish embryos instead of the one-cell stage fertilized eggs or the 6 hpf gastrulae aiming to recapitulate the human response to the drugs with more specificity.

For mechanistic studies with larvae exposed to drugs, we chose a maximum CsA dose of 5 μM to avoid severe body deformity or lethality. We also began treatment to the drugs when the larval age was 72 hpf. The 24 h treatment of the 72 hpf larvae that have a functional liver was intended not only to generate quantifiable moderate toxic endpoints but also to explore whether there was any alteration in the specific drug-metabolizing CYP enzyme. Exposure of 72 hpf larvae to 10 μM CsA only has been shown to cause significant behavioral defects but no morphological anomalies (Clift *et al.*, 2015). Consistent with this report, our current study showed that 10 μM CsA-treated larvae developed like control larvae. With 5 μM CsA, there were no morphological defects and the treated larvae were similar to controls (data not shown). However, with ketamine and CsA (0.5–5.0 μM) co-treatment, the larvae developed pericardial edema that was not present in the controls or in the ALCAR co-treated larvae suggesting that CsA accentuated the toxicity triggered by ketamine.

We then explored potential changes in the expression of the specific drug metabolizing CYP enzyme as xenobiotic drugs given concomitantly might compete for the same CYP binding sites resulting in inhibition of the metabolism of co-administered drugs

(Hollenberg, 2002). In humans, CP450 3A4 (CYP3A4) is the predominant CYP isoform in the liver and small intestine (Paine *et al.*, 1997). In 2005, a full-length CYP3A cDNA from zebrafish was cloned, which was named as CYP3A65 (Tseng *et al.*, 2005) by the Cytochrome P450 Nomenclature Committee. The expression pattern and xenobiotic sensitivity of CYP3A65 in the zebrafish early life stages are similar to mammalian CYP3A4 (Chng *et al.*, 2012; Tseng *et al.*, 2005). Many xenobiotics, including rifampicin, dexamethasone and phenobarbital induce CYP3A4 gene expression in mammals (Pichard *et al.*, 1990) and CYP3A65 in zebrafish (Tseng *et al.*, 2005). Our current study showed that ketamine and CsA dose-dependently induced CYP3A65 expression. When combined, ketamine and various doses of CsA induced significantly increased CYP3A65 expression in the larvae compared to control. Ketamine co-treatment with 2 and 5 μM CsA doses resulted in further increase in CYP3A65 expression suggesting that the adverse effects caused by ketamine/CsA co-treatment may not be the consequence of slower drug metabolism in the larvae. This concept was further supported by the observation that ALCAR inhibited the upregulation in CYP3A65 expression induced by either ketamine or ketamine/CsA and normalized its expression to control levels. Together these results suggested that toxicities caused by ketamine/CsA drug interaction might not be due to slower drug metabolism as CYP3A65 expression was upregulated, although hepatic dysfunction due to abnormally high CYP3A65 induction can potentially be a concern. It is also important to consider the fact that ketamine is metabolized enantio-selectively to norketamine (Schmitz *et al.*, 2010), which is pharmacologically active as an NMDA receptor antagonist (Woolf and Adams, 1987) and is capable of inducing toxicity (Lin *et al.*, 2014). Therefore, enhanced metabolism of ketamine may still be a factor in toxicities observed in the larvae

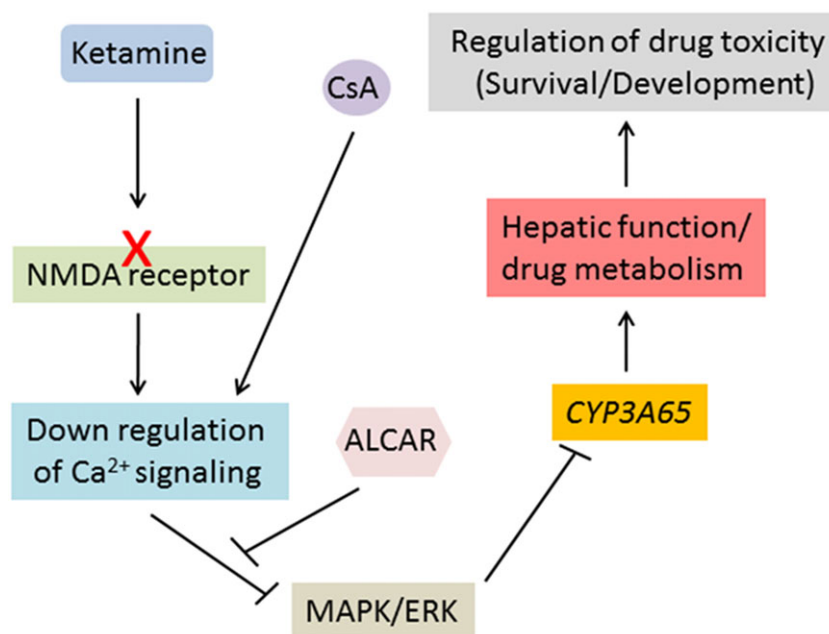


Figure 7. Schematic presentation of a potential mechanism of ALCAR's protective effects against ketamine/CsA toxicity in zebrafish larvae. In the zebrafish embryos, ketamine as an antagonist of NMDA receptors is known to suppress MAPK/ERK, which is normalized in the presence of ALCAR. Both ketamine and CsA as antagonists of Ca^{2+} signaling could additively suppress MAPK/ERK activity. There is an inverse relationship between MAPK/ERK activity and CYP3A4 (zebrafish CYP3A65) expression. Potentially, ALCAR by normalizing ketamine-induced MAPK/ERK suppression results in the expression of CYP3A65 at the control level, which could contribute to ALCAR's beneficial effects on ketamine toxicity that is further exacerbated by CsA. ALCAR, acetyl-L-carnitine; CsA, cyclosporine A; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NMDA, N-methyl-D-aspartate. [Colour figure can be viewed at wileyonlinelibrary.com]

treated with ketamine/CsA. Whether norketamine affects other targets usually not affected by ketamine needs to be determined, although, if true, can explain the more pronounced ketamine/CsA toxicity compared to ketamine toxicity alone.

Our previous studies showed a potential mechanism for the effects of ketamine and ALCAR on mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) linked to Ca^{2+} in zebrafish (Guo *et al.*, 2017; Kanungo *et al.*, 2012). Furthermore, a link between reduction in MAPK/ERK activity and ketamine-induced neurotoxicity in mice has also been reported (Straiko *et al.*, 2009). Chemical inhibition of MAPK/ERK signaling increased CYP3A4 expression in human liver cells (Woolsey *et al.*, 2013). As ketamine inhibits MAPK/ERK signaling in the zebrafish embryos, which is countered by ALCAR (Kanungo *et al.*, 2012), it is possible that ketamine-induced reduction in MAPK/ERK signaling upregulated CYP3A65 expression (Fig. 7). CsA has been shown to suppress MAPK/ERK activity in human K562 cells (Sawafuji *et al.*, 2003). Both ketamine and CsA as inhibitors of Ca^{2+} signaling suppress MAPK/ERK activity in rat brains (Zhao *et al.*, 2008). We have previously reported that ketamine-induced toxicity could involve reduced intracellular Ca^{2+} as ketamine antagonizes the Ca^{2+} -permeable NMDA receptors (Guo *et al.*, 2017; Kanungo *et al.*, 2012). In this context, the effects of ketamine and CsA together on the inhibition of Ca^{2+} signaling could potentially be additive resulting in further suppression of MAPK/ERK leading to the upregulation of CYP3A65 expression (Fig. 7). A direct role of increased CYP3A65 expression in ketamine/CsA drug interaction-induced toxicities in the zebrafish larvae needs to be determined using various inhibitors of CYP3A65 to reverse the toxicity. Nonetheless, the current study provides for the first time an insight into the mechanism of ketamine/CsA combination toxicity *in vivo* by reversing it with ALCAR that could help understand human conditions resulting from drug–drug interaction.

Conflict of interest

The authors did not report any conflict of interest.

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