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Acetyl L-carnitine targets adenosine triphosphate synthase in protecting zebrafish embryos from toxicities induced by verapamil and ketamine: An *in vivo* assessment

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Abstract

Verapamil is a Ca^{2+} channel blocker and is highly prescribed as an anti-anginal, antiarrhythmic and antihypertensive drug. Ketamine, an antagonist of the Ca^{2+} -permeable *N*-methyl-D-aspartate-type glutamate receptors, is a pediatric anesthetic. Previously we have shown that acetyl L-carnitine (ALCAR) reverses ketamine-induced attenuation of heart rate and neurotoxicity in zebrafish embryos. Here, we used 48 h post-fertilization zebrafish embryos that were exposed to relevant drugs for 2 or 4 h. Heart beat and overall development were monitored *in vivo*. In 48 h post-fertilization embryos, 2 mM ketamine reduced heart rate in a 2 or 4 h exposure and 0.5 mM ALCAR neutralized this effect. ALCAR could reverse ketamine's effect, possibly through a compensatory mechanism involving extracellular Ca^{2+} entry through L-type Ca^{2+} channels that ALCAR is known to activate. Hence, we used verapamil to block the L-type Ca^{2+} channels. Verapamil was more potent in attenuating heart rate and inducing morphological defects in the embryos compared to ketamine at specific times of exposure. ALCAR reversed cardiotoxicity and developmental toxicity in the embryos exposed to verapamil or verapamil plus ketamine, even in the presence of 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester, an inhibitor of intracellular Ca^{2+} release suggesting that ALCAR acts via effectors downstream of Ca^{2+} . In fact, ALCAR's protective effect was blunted by oligomycin A, an inhibitor of adenosine triphosphate synthase that acts downstream of Ca^{2+} during adenosine triphosphate generation. We have identified, for the first time, using *in vivo* studies, a downstream effector of ALCAR that is critical in abrogating ketamine- and verapamil-induced developmental toxicities. Published 2016. This article is a U.S. Government work and is in the public domain in the USA.

Keywords

verapamil; heart rate; ketamine; zebrafish; acetyl L-carnitine; developmental toxicity

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Conflict of interest

The authors did not report any conflict of interest.

Introduction

Verapamil belongs to the phenylalkylamine class of L-type Ca^{2+} channel blockers and is used to treat cardiac arrhythmias, hypertension and angina (Prisant, 2001). Overdoses of Ca^{2+} channel blockers cause rapid development of hypotension, bradycardia and cardiac arrest (Derlet and Horowitz, 1995). 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). A number of studies on rodents and non-human primates have shown that treatment with high doses of ketamine or exposure for long durations during susceptible periods of development (the brain growth spurt) can induce abnormally high levels of neuroapoptosis (Haberny *et al.*, 2002; Ikonomidou *et al.*, 1999; Lahti *et al.*, 2001; Larsen *et al.*, 1998; Malhotra *et al.*, 1997; Maxwell *et al.*, 2006; Slikker *et al.*, 2007; Wang *et al.*, 2006). Studies in humans and animals have shown that ketamine has a direct negative (Aya *et al.*, 1997; Graf *et al.*, 1995; Hara *et al.*, 1994; Yeh *et al.*, 1994) or an indirect positive (Cook *et al.*, 1991; Endou *et al.*, 1992; Riou *et al.*, 1990) inotropic effect on myocardial contractility. Ketamine reduces heart rate in rats (Zausig *et al.*, 2009) and guinea pigs (Kim *et al.*, 2006) and acts as a cardiac depressant in dogs after pharmacological blockade of the autonomic nervous system (Pagel *et al.*, 1992). In rhesus monkeys, extended intravenous infusions of ketamine result in decreased heart rates in pregnant females and in postnatal day 5 and 35 infants (Hotchkiss *et al.*, 2007). However, the mechanism of how ketamine impacts cardiac function is not clear.

Several drugs that either directly or indirectly inhibit mitochondrial fatty acid oxidation (e.g., etomoxir, oxfenicine, dichloroacetate, trimetazidine, ranolazine, pyruvate, malonyl-coenzyme A decarboxylase inhibitors), improve cardiac function in experimental models and humans with cardiac ischemia and failure (Lopaschuk, 2002; Stanley, 2005). Acetyl L-carnitine (ALCAR) is an L-lysine derivative and its main role lies in the transport of long chain fatty acids into mitochondria to enter the β -oxidation cycle (Bohles *et al.*, 1994). ALCAR preserves the mechanical function of ischemic swine hearts perfused in the presence of free fatty acids (Liedtke and Nellis, 1979). Moreover, ALCAR has been shown to ameliorate ketamine-induced behavioral alterations and body weight deficits in rats (Boctor *et al.*, 2008), in addition to having neuroprotective effects in mammals (Zou *et al.*, 2008), and both neuroprotective and cardioprotective effects in zebrafish (Cuevas *et al.*, 2013; Kanungo *et al.*, 2012).

The zebrafish (*Danio rerio*) embryo has become an important experimental model for studying various diseases, protein functions, drug toxicity and various physiological responses (Kanungo *et al.*, 2014; Vogel, 2000). Accordingly, the therapeutic and toxic responses for both cardiac and non-cardiac drugs can be assessed by simply immersing the embryos in the water with the test drug (Kanungo *et al.*, 2012; Langheinrich *et al.*, 2003; Milan *et al.*, 2003). We have previously shown that consistent with its effects on mammals (Hotchkiss *et al.*, 2007; Irifune *et al.*, 1991, 1997; Kim *et al.*, 2006; Zausig *et al.*, 2009), in zebrafish embryos, ketamine modulates the serotonergic and dopamine systems (Robinson *et al.*, 2015, 2016) and heart rate (Kanungo *et al.*, 2012). Based on reported observations that

verapamil affects cardiac function in mammals including humans (Derlet and Horowitz, 1995; Prisant, 2001), the goal of this study was to test whether verapamil has similar effects and ketamine accentuates verapamil's effects in zebrafish, an emerging alternate vertebrate animal model for drug screening and drug safety assessments. Here, we have undertaken *in vivo* mechanistic studies to demonstrate how ALCAR reverses ketamine- and verapamil-induced cardiac and developmental toxicities.

Materials and methods

Animals (zebrafish)

Adult wild-type zebrafish (*D. 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 eight 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 zebrafish 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). ALCAR, oligomycin A (oligomycin) and TMB-8 (3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester) were purchased from Sigma (St. Louis, MO, USA). Verapamil hydrochloride was purchased from TOCRIS Biosciences (Ellisville, MO, USA). All other reagents including dinitrophenol used in this study were purchased from Sigma unless mentioned otherwise. Verapamil (0.05 M) and ALCAR stock (1 M) solutions were made fresh with buffered egg water. Stocks of oligomycin A (10 mM), TMB-8 (0.5 M) and dinitrophenol (1 M) were prepared using dimethyl sulfoxide as the solvent.

Treatment of zebrafish embryos with verapamil, ketamine, acetyl L-carnitine and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester

For treatment with verapamil, ketamine, ALCAR, TMB-8 and oligomycin A, 48 h post-fertilization (hpf) manually dechorionated embryos were used. For every experiment, in each treatment, 10 embryos were placed in the six-well plates (Nunc non-treated plates) with 5 ml buffered egg water. Ketamine (2.0 mM), verapamil (10 µM–1 mM), ALCAR (0.5 mM), TMB-8 (0.25–0.5 mM) and oligomycin A (0.25–5.0 µM) treatments, alone or in combination continued for 2 or 4 h (static exposures). Control groups treated with equivalent amount of vehicle (dimethyl sulfoxide) were examined in parallel. As we previously

reported, ALCAR at 0.5 mM did not have any effect on heart rate at 2 and 4 h of exposure. In the earlier report we also showed that 0.1 mM ALCAR did not affect the ketamine-induced decrease in heart rate, doses of 0.5 and 1.0 mM ALCAR were equally effective in completely inhibiting that effect (Kanungo *et al.*, 2012). Based on these data we chose to use 0.5 mM ALCAR for all our experiments in the present study. For the ketamine dose, we have earlier determined that 2 mM ketamine exposure results in an internal exposure of the anesthetic level of plasma concentrations in humans (Robinson *et al.*, 2015; Trickler *et al.*, 2014). For co-exposure to various drug combinations, the drugs were added together at the same time. For acute toxicity assessments, drug exposure continued for 2 or 4 h.

Embryo imaging, scoring heart rate and measurement of the pericardial sac area

Live images of the embryos in the six-well plates were acquired using an Olympus SZX 16 binocular microscope and DP72 camera and heart rate of each embryo was measured by visually counting ventricular beats per minute on the live video using a timer (stop watch). The data are presented by converting heart beats per minute to percentage of control (100%). Data for each group ($n = 10$) were used to calculate the mean and standard deviation (SD). The extent of pericardial edema in the embryos was determined by quantitation of the pericardial sac area (Carney *et al.*, 2004). The pericardial sac area was outlined in lateral view images of the embryos and the area was quantitated using the DP2 BSW microscope digital camera software (Olympus, Tokyo, Japan). The statistical significance of the effects of the various treatments on heart rate and pericardial edema was determined by one-way ANOVA (Sigma Stat) using Holm–Sidak pairwise multiple comparison *post-hoc* analysis.

Results

To assess the effects of verapamil on heart rate, we used zebrafish embryos at 48 hpf (Fig. 1). In these embryos, treatment with various doses of verapamil altered heart rate dose-dependently in 2 and 4 h post-treatment (Fig. 1). Doses up to 20 μ M did not have any significant effect, whereas doses 50 μ M and above significantly reduced heart rate. Ketamine decreases Ca^{2+} release from intracellular stores of rat ventricular myocytes (Kanaya *et al.*, 1998) and its cardiac depressive effects could be caused by interference with Ca^{2+} influx (Endou *et al.*, 1992; Kunst *et al.*, 1999). L-carnitine, a naturally occurring amino acid that induces fatty acid oxidation, neutralizes ketamine-induced toxicities, such as neuronal apoptosis in the developing rat frontal cortex (Zou *et al.*, 2008). In our previous studies, we had shown that ALCAR counteracted ketamine-induced attenuation of heart rate, implying that ALCAR potentially acted to restore the intracellular Ca^{2+} levels that could have been reduced due to NMDA receptor blockade by ketamine. Here, we hypothesized that were it a plausible scenario, ALCAR would also counteract verapamil (an L-type Ca^{2+} channel inhibitor)-induced cardiotoxicity. We chose to use a verapamil dose, 150 μ M, which could cause ~50% reduction in heart rate in 2 h of exposure. At this dose, as expected, verapamil reduced heart rate at 2 and 4 h (Fig. 2). ALCAR reversed the effects of not only 150 μ M verapamil but also of a higher dose of verapamil (500 μ M) (Fig. 2).

It has been reported that pretreatment of surgical patients with verapamil may reduce the dose of ketamine required for anesthesia due to increased duration of sleeping time and can

potentiate the effects of some central nervous system drugs such as ketamine in mice. The latter effect has been attributed to blocked neuronal Ca^{2+} channels although ketamine does not interact with verapamil binding site on the L-type Ca^{2+} channels. We tested whether zebrafish embryos also reproduce similar effects observed in mammals. In our experiments, we used an anesthetic dose of ketamine (2 mM in water) and co-treated 48 hpf embryos with increasing doses of verapamil (50 μM –1.0 mM). The results showed that verapamil significantly potentiated ketamine's effects on the attenuating heart rate (Fig. 3). These results indicated the effects of ketamine and verapamil were additive on the heart rate due to blockade of Ca^{2+} entry through NMDA receptors and the L-type Ca^{2+} channels by the drugs, respectively.

To examine whether in the presence of ALCAR (0.5 mM), the effects of ketamine plus verapamil on the heart rate could be completely reversed, as ALCAR could reverse the effects of ketamine and verapamil alone, we treated the embryos with all three drugs. The results showed that ALCAR indeed was effective in restoring the attenuated heart rate induced by ketamine and verapamil together to the control level (Fig. 3). These results further suggested that ALCAR could potentially induce intracellular Ca^{2+} release to compensate for the blockade of Ca^{2+} entry through the NMDA receptors and the L-type Ca^{2+} channels by ketamine and verapamil, respectively and thus could circumvent the drop in heart rate due to Ca^{2+} deprivation.

In a previous study, we showed that ketamine (2 mM with 20 h exposure) acted as a developmental toxicant in zebrafish. However, when treated for 2–4 h, ketamine's developmental toxicity at the 2 mM dose was not apparent compared to control (Fig. 4A,B), although contrary to controls (Fig. 5A), the pericardial edema was evident in the ketamine-treated embryos (Fig. 4B). With 1 mM verapamil, a number of embryos showed specific deformities, particularly curved tails ($41.2 \pm 3.3\%$) (Fig. 4C) and severe pericardial edema (Fig. 5C) and within minutes, their hearts would stop beating, subsequently leading to death (data not shown). However, embryos treated with verapamil (0.1 or 0.2 mM) and 2 mM ketamine did not exhibit the curved tail phenotype (Fig. 4D,E) indicating that the effect was specific to the 1 mM verapamil dose. Embryos treated with 2 mM ketamine and 1 mM verapamil were more adversely affected (Figs. 4F and 5D) than those treated with 1 mM verapamil alone (Figs. 4C and 5C) and would eventually die faster than the latter (data not shown). A number of these embryos also had curved tails ($41.5 \pm 3.4\%$) (Fig. 4F) similar to the group treated with 1 mM verapamil alone (Fig. 4C). Embryos treated with 2 mM ketamine do not die even when the static exposure continues for a few days (data not shown). However, in the presence of 0.5 mM ALCAR, none of the effects of verapamil alone (Fig. 4G) or with ketamine (Figs. 4H and 5E) were observed indicating that ALCAR reversed the toxic effects of ketamine and verapamil as it did for the heart rate. The extent of the edematous pericardia is quantitatively presented showing the adverse effects of ketamine and verapamil alone and in combination (Fig. 5F).

As ALCAR prevented ketamine- and verapamil-induced cardiotoxicity, we hypothesized that intracellular Ca^{2+} release could compensate for the lack of extracellular Ca^{2+} via NMDA receptors (by ketamine) and L-type Ca^{2+} channels (by verapamil). Therefore, we used the intracellular Ca^{2+} release inhibitor TMB-8 to block such a compensatory potential.

TMB-8 modulates cellular Ca^{2+} metabolism by preventing intracellular Ca^{2+} mobilization via inhibition of the inositol 1,4,5-trisphosphate-induced Ca^{2+} release (Nishizuka, 1988). In zebrafish embryos, TMB-8 dose-dependently reduced heart rate (data not shown) and a higher dose (0.5 mM) completely caused the heart to stop beating in 2 h of co-exposure with either ketamine or verapamil alone or in combination (Fig. 6). However, in the presence of ALCAR, the adverse effect of TMB-8 was completely obliterated. These data suggested that ALCAR function is potentially carried out by an effector molecule downstream of Ca^{2+} .

With the reversal of heart rate to control levels by ALCAR even in the presence of ketamine, verapamil and TMB-8, the latter three inhibiting intracellular Ca^{2+} , we redirected our focus to selective targets downstream of Ca^{2+} . One of the major downstream effectors of Ca^{2+} is adenosine triphosphate (ATP) synthase. The addition of oligomycin A (an inhibitor of the mitochondrial F-ATP synthase) to embryos along with ALCAR, ketamine and verapamil obliterated the protective effects of ALCAR on ketamine- and verapamil-induced cardiotoxicity (Fig. 7). Doses of oligomycin less than 0.5 mM were ineffective in interfering with ALCAR's beneficial effects (data not shown). These results indicated that ALCAR's function is not dependent on Ca^{2+} but is mediated by the latter's downstream target, ATP synthase.

Discussion

Our previous study has shown that ALCAR reverses ketamine-induced attenuated heart rate in zebrafish embryos and we hypothesized that ALCAR, due to its ability to upregulate L-type Ca^{2+} channels (Tewari *et al.*, 1995), could compensate for ketamine-induced blockade of Ca^{2+} -permeable NMDA receptors (Kanungo *et al.*, 2012) (Fig. 8). It has been shown that ketamine decreases Ca^{2+} release from intracellular stores of rat ventricular myocytes (Kanaya *et al.*, 1998). There are also reports that the cardiac depressive effects of ketamine could be caused by interference with Ca^{2+} influx (Endou *et al.*, 1992; Kunst *et al.*, 1999). It has also been suggested that pretreatment of surgical patients with verapamil may reduce the dose of ketamine required for anesthesia due to increased duration of sleeping time (Saha *et al.*, 1990) and can potentiate the effects of some central nervous system drugs such as ketamine in mice (Shen and Sangiah, 1995). The latter effect has been attributed to blocked neuronal Ca^{2+} channels although ketamine does not interact with the verapamil binding site on the L-type Ca^{2+} channels (Hirota and Lambert, 1996). In the current study, we provide evidence that ALCAR can reverse verapamil-induced cardiotoxicity caused by verapamil treatment alone or with ketamine. Verapamil is an L-type Ca^{2+} channel blocker (Prisant, 2001), and we show that verapamil dose-dependently attenuated the heart rate in zebrafish embryos. At high dose, it induced severe developmental anomalies and cardiotoxicity. With these results, what remained unanswered however, is whether in the presence of all three compounds, verapamil, ketamine and ALCAR, normalization of overall development and heart rate occurs or not. To answer this question, we followed a completely non-invasive *in vivo* approach. Instead of directly monitoring Ca^{2+} in the heart by imaging, which would need microinjection of fluorescent Ca^{2+} trackers, and therefore, may interfere with survival rate and normal physiology in addition to being exposed to the drugs of interest, we chose to use various inhibitors of Ca^{2+} and monitored the downstream physiological effect on the heart rate. The zebrafish embryo is an intact organism in which the heart beat is observed in

its native environment (water) rather than in isolation in a culture medium (Kim *et al.*, 2006), an advantage that can be utilized to test for safety and efficacy of drugs *in vivo*.

The effect of ketamine, an NMDA receptor antagonist, on heart rate has been reported for a variety of mammals, such as rats (Zausig *et al.*, 2009), rabbits (Yeh *et al.*, 1994) and guinea pigs (Kim *et al.*, 2006). The zebrafish embryo response to ketamine with regard to heart rate is also concordant with that of human infants (Saarenmaa *et al.*, 2001) and non-human primate infants at postnatal days 5 and 35 (Hotchkiss *et al.*, 2007). Zebrafish embryos can accurately model drug effects on mammals and humans (Kanungo *et al.*, 2014), and our earlier studies have shown that in 48 hpf embryos, a 2 h exposure to ketamine was effective in significantly lowering the heart rate (Kanungo *et al.*, 2012).

In humans, neonatal myocardium appears more dependent on the entry of extracellular Ca^{2+} rather than the release of Ca^{2+} from intracellular sarcoplasmic reticulum (Kudoh *et al.*, 2003). Ketamine decreases Ca^{2+} release from intracellular stores of rat ventricular myocytes (Kanaya *et al.*, 1998). There are also reports that the cardiac depressive effects of ketamine could be caused by interference with Ca^{2+} influx (Endou *et al.*, 1992; Kunst *et al.*, 1999). Among the compounds that appear to have a neutralizing effect on ketamine-induced toxicities, such as neuronal apoptosis in the developing rat frontal cortex, is L-carnitine (Zou *et al.*, 2008), a naturally occurring amino acid that induces fatty acid oxidation. The mechanism underlying the effect of L-carnitine to inhibit such neurotoxicity is not clear. On the other hand, ketamine is known to induce blockade of lipid oxidation in rat heart (Ovchinnikov *et al.*, 1989). Here, we show that ALCAR not only neutralized the ketamine-induced decrease in heart rate as we reported in our earlier study (Kanungo *et al.*, 2012), but also reversed verapamil's adverse effects on the heart rate in the presence or absence of ketamine, suggesting that ALCAR may have altered cellular Ca^{2+} transport or intracellular Ca^{2+} release or both. Carnitine is known to be an endogenous promoter of Ca^{2+} efflux from rat heart mitochondria (Baydoun *et al.*, 1988) and acyl-carnitines increase Ca^{2+} concentrations in the cytoplasm of rat ventricular cardiomyocytes (Berezhnov *et al.*, 2008). In patients with mild diastolic heart failure, ALCAR improves their symptoms (Serati *et al.*, 2010). Differential effects of ketamine and ALCAR have been reported, which show that 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 (Hagen *et al.*, 2002). Although our study shows the differential effects of ketamine (with or without verapamil) and ALCAR on the zebrafish heart rate, direct evidence on whether such counteracting effects are exclusively mediated by ATP, remains to be uncovered. Our studies showed that ALCAR completely reverses the adverse effects on heart rate (even lethality) induced by 0.5 mM dinitrophenol alone or with ketamine co-treatment (data not shown). These findings suggest that ALCAR counteracts acute cardiotoxicity in the zebrafish embryos by replenishing ATP in a way that is not dinitrophenol-sensitive. This would mean that ATP production might be sustained via beta-oxidation of fatty acids instead of the oxidative phosphorylation. ALCAR can induce fatty acid metabolism via beta-oxidation (Hagen *et al.*, 2002).

In the current study, ALCAR also reversed the reduced heart rate induced by verapamil, an L-type Ca^{2+} channel blocker, in the zebrafish embryos even in the presence of ketamine,

indicating that ALCAR may be regulating heart rate by modulating intracellular Ca^{2+} levels. In our *in vivo* approach to modulate the availability of cellular Ca^{2+} to determine how ketamine, verapamil and ALCAR modulate heart rate, we used the intracellular Ca^{2+} inhibitor, TMB-8. TMB-8 acts as an intracellular Ca^{2+} antagonist. In mammalian skeletal or smooth muscle, TMB-8, an amphiphilic ester, acts to inhibit intracellular Ca^{2+} availability (Chiou and Malagodi, 1975; Malagodi and Chiou, 1974a,b). TMB-8 enhanced ketamine inhibition of inositol 1,4,5-trisphosphate production in rat heart myocytes (Kudoh *et al.*, 2002). Our study showed that just like verapamil, TMB-8 alone could drastically reduce heart rate followed by lethality (data not shown) and its effects were exacerbated in the presence of ketamine; however, it was ineffective in altering heart rate or overall development in the presence of ALCAR alone or in combination with ketamine and verapamil, suggesting that reversal of this acute toxicity depended on targets acting downstream of Ca^{2+} .

It has been reported that verapamil has the potential to change myocardial Ca^{2+} sensitivity in zebrafish and the mechanism by which this occurs may involve myofilament Ca^{2+} sensitivity of the heart muscle (Haustein *et al.*, 2015). 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 was discussed in our previous study (Kanungo *et al.*, 2012). Involvement of MAPK/ERK pathway in ketamine-induced neurotoxicity in mice has been reported (Straiko *et al.*, 2009). However, a distinct effector molecule downstream of the MAPK/ERK signaling pathway, has not been identified that can explain ketamine-induced toxicity, in which case intervention at the MAPK/ERK level might prove deleterious as this signaling pathway is ubiquitous and critical for overall cellular physiology.

We were focused on identifying the effector(s) downstream of Ca^{2+} signaling that could be activated by ALCAR. In muscle-derived cell cultures from patients with collagen type VI myopathies, addition of oligomycin results in mitochondrial depolarization (Angelin *et al.*, 2007). In the present investigation, we show that oligomycin blunted ALCAR's beneficial effects on toxicities induced by either ketamine or verapamil or TMB-8 alone or all in combination. Oligomycin A, an inhibitor of the mitochondrial F-ATP synthase, blocks ATP synthesis in the mitochondria. Our results thus point to a direct role of ALCAR-mediated ATP synthase activity in protecting zebrafish embryos from toxicities induced by ketamine, verapamil and TMB-8, alone or in combination (Fig. 8). Our future studies would focus on how ALCAR affects ATP synthase activity, whether at the transcriptional or post-translational levels or by upregulating the expression of any inhibitor(s) of ATPase. Nonetheless, for the first time, we have identified a molecule that is responsible for imparting ALCAR's protective effects on ketamine- and verapamil-induced toxicities. These results provide mechanistic data with an insight on how these drugs might work in humans and how their reported toxicities could be ameliorated with potential intervention.

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Figure 1.

Effect of verapamil on heart rate in zebrafish embryos. Embryos (48 hpf) were exposed to various doses of verapamil for 2 or 4 h. Each group consisted of 10 embryos and the experiment was repeated at least three times. Heart rate was measured in beats per minute and the data from all the embryos in a group were averaged and shown as mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between the control and the treated groups. Significance (*) was set at $P < 0.05$.

Figure 2.

Effects of verapamil and ALCAR on heart rate in zebrafish embryos. Embryos (48 hpf) were exposed to various doses of verapamil alone or with 0.5 mM ALCAR. Each exposure (static exposure) group consisted of 10 embryos and the experiment was repeated at least three times. Heart rate was measured in beats per minute and the data from all the embryos in a group were averaged and shown as mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between the control and the treated groups. Significance (*) was set at $P < 0.05$. Heart rates are shown for 2 and 4 h exposures. ALCAR, acetyl L-carnitine.

Figure 3.

Effects of verapamil and ketamine in the presence or absence of ALCAR on heart rate in zebrafish embryos. Embryos (48 hpf) were exposed to various doses of verapamil alone or with 2 mM ketamine and in the presence of 0.5 mM ALCAR. Each exposure group consisted of 10 embryos and the experiment was repeated at least three times. Heart rate, at 2 and 4 h post-exposure, was measured in beats per minute and the data from all the embryos in a group were averaged and shown as mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between the control and the treated groups. Significance (*) was set at $P < 0.05$. ALCAR, acetyl L-carnitine.

Figure 4.

Morphology of 48 hpf zebrafish embryos treated with verapamil or ketamine or both, in the presence or absence of ALCAR for 2 h. Group pictures of embryos show control (A), and groups treated with 2 mM ketamine (B), 1 mM verapamil (C), 2 mM ketamine with 100 μ M verapamil (D), 2 mM ketamine with 200 μ M verapamil (E), 2 mM ketamine with 1 mM verapamil (F), 1 mM verapamil with 0.5 mM ALCAR (G) and 2 mM ketamine with 1 mM verapamil and 0.5 mM ALCAR (H). Curved tails are indicated (*). ALCAR, acetyl L-carnitine; Ket, ketamine; Ver, verapamil.

Figure 5.

Morphology showing pericardial area of 48 hpf zebrafish embryos treated with verapamil or ketamine or both in the presence or absence of ALCAR for 2 h. Control (A) and embryos treated with 2 mM ketamine (B), 1 mM verapamil (C), 2 mM ketamine with 1 mM verapamil (D) and 2 mM ketamine with 1 mM verapamil and 0.5 mM ALCAR (E). Arrows indicate the pericardium. Pericardial area is indicated by dotted lines and the pericardial area measurements are shown (F). Values are averaged and shown as mean $\pm$ SD. One-way ANOVA was used to compare the pericardial areas between the control and the treated groups. Significance (*) was set at $P < 0.05$. Bar = 125 μ M. ALCAR, acetyl L-carnitine.

Figure 6.

TMB-8, an inhibitor of intracellular Ca^{2+} release does not interrupt ALCAR's protection from ketamine- and verapamil-induced cardiotoxicity. Embryos (48 hpf) were exposed for 2 h with 1 mM verapamil alone or with 2 mM ketamine and in the presence of 0.5 mM ALCAR and TMB-8 (either 0.25 or 0.5 mM). Each exposure group consisted of 10 embryos and the experiment was repeated three times. Post-exposure, heart rate was measured in beats per minute and the data from all the embryos in a group were averaged and shown as mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between the control and the treated groups. Significance (*) was set at $P < 0.05$. ALCAR, acetyl L-carnitine; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester.

Figure 7. Oligomycin disrupts ALCAR's protective effects on ketamine-and verapamil- induced cardiotoxicity. Embryos (48 hpf) were exposed for 2 h with 1 mM verapamil alone or with 2 mM ketamine and in the presence of 0.5 mM ALCAR, and oligomycin (5 μM). Each exposure group consisted of 10 embryos and the experiment was repeated three times. Post-exposure, heart rate was measured in beats per minute and the data from all the embryos in a group were averaged and shown as mean ± SD. One-way ANOVA was used to compare the heart rates between the control and the treated groups. Significance (*) was set at $P < 0.05$. ALCAR, acetyl L-carnitine.

Figure 8.

Schematic presentation of a potential mechanism of ALCAR's reversal of ketamine- and verapamil-induced cardiotoxicity. NMDA receptors are Ca²⁺ permeable. Ketamine as an antagonist of NMDA receptors can inhibit Ca²⁺ entry into the cells. In the absence of intracellular Ca²⁺, the mitochondrial ATP synthase activity is inhibited that could result in blunted ATP generation. ALCAR potentially bypasses the need for Ca²⁺ to induce ATP synthase activity and instead directly induces ATP synthase activity. ALCAR, acetyl L-carnitine; ATP, adenosine triphosphate; NMDAR, *N*-methyl-D-aspartate receptor.