Toxicology Reports 8 (2021) 1300–1304

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Investigation of the protective effect of acetazolamide and SLC-0111 on carbon tetrachloride-induced toxicity in fruit fly

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ARTICLE INFO

Handling Editor Dr. Aristidis Tsatsakis

Keywords:
Carbon tetrachloride
Drosophila melanogaster
Longevity
Malondialdehyde
Superoxide dismutase

ABSTRACT

Sulfonamide-based compounds in the development of drugs used in cancer treatment have been started to be investigated recently. In the current work, it was determined the protective effect of Acetazolamide (AAZ) and SLC-0111 on carbon tetrachloride-induced toxicity in the fruit fly (*Drosophila melanogaster*). AAZ and SLC-0111 were used as a nonselective and selective inhibitor of carbonic anhydrase isozymes, respectively, to compare the selectivity effect of drugs on toxicity. The experimental toxicity was created by carbon tetrachloride (CCl_4) that causes tissue damage to the first stage larvae of fruit fly and used as a model organism. The effect of AAZ and SLC-0111 on toxicity of insect survival, sex ratio, longevity and some biochemical parameters such as Malondialdehyde-MDA content, Superoxide dismutase-SOD and Glutathione-*S*-transferase-GST activity were tested. According to the data obtained, feeding of insects with AAZ and SLC-0111 (2.5 and 10 mM, respectively) affected their survival and development positively against the toxicity induced by CCl_4. Compared to the control group, GST and SOD activity was higher in pups and adults (SLC-0111 < AAZ). Because of this study, SLC-0111 is thought to be useful in protecting against the harmful effects of reactive oxygen species.

1. Introduction

Cancer is a disease associated with clonal evolution and cell competition within the body and is the second most common leading cause of death worldwide. New drug research for cancer treatment has gained increasing attention recently. Studies show that investments in cancer treatments and research are financially costly but contribute a small percentage to patients' recovery [1]. More recent studies have shown that some carbonic anhydrase (CA) isozymes [an enzyme that catalyzes the physiologically simple, but critical reaction that is the conversion of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) and proton (H^+) ions], specifically membrane-bound isozymes CA IX and CA XII, are overexpressed in most cancer types and are related to hypoxic tumor progression and invasion [2–4]. Therefore, selective inhibition of these tumor-overexpressed isoforms constitutes a viable strategy to find more efficient and potent cancer drugs to treat metastatic tumors. However, potent first-generation CA inhibitors available clinically, such as acetazolamide (AAZ), are unideal in cancer treatment due to lack of selectivity against CA IX and CA XII [5,6]. Alternatively, SLC-0111, one of the small molecules that selectively inhibit CA IX and CA XII, recently entered phase II clinical trials as the first CA inhibitor for treating advanced metastatic solid tumors [7,8].

Reactive oxygen species (ROS) can occur because of normal metabolism, as well as chemicals, drugs, diseases, and aging. There is an increase in ROS in cells undergoing apoptosis during cancer formation. For example, the phase 2 detoxifying enzymes Glutathione-*S*-transferase (GST) and superoxide dismutase (SOD) primarily affect the removal of carcinogens by helping reduce the ROS that leads to apoptosis [9]. When the antioxidant balance in the body shifts in favor of oxidants, free radicals interact with fatty acids to form peroxidation products. Malondialdehyde (MDA), an indicator of lipid peroxidation, is considered an indicator of free radical level. Carbon tetrachloride (CCl_4) is a peroxidant that leads to experimental liver degeneration and exhibits oxidative properties by combining unsaturated fatty acids above a certain dose [10,11].

The fruit fly *Drosophila melanogaster* Meigen is a simple and important organism used as a model of neurodegenerative/metabolic diseases and cancer [12–14]. *D. melanogaster* is used, considering the information obtained from insect models, inferences are made for mammalian models [12,14–16]. Previous studies were evidence that the models can be used to identify cancer therapeutics [17]. For example, *D. melanogaster*' fat tissue works similarly to mammalian fat tissue and

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https://doi.org/10.1016/j.toxrep.2021.06.014
Received 9 February 2021; Received in revised form 12 April 2021; Accepted 14 June 2021
Available online 16 June 2021

liver [18,19]. The adult and larval stages of *D. melanogaster* in the feeding phase are used in cancer models [14]. For this reason, the effect of selected chemicals on fat tissue was investigated by inducing toxicity in the target insects. An in vivo investigation of the effect of AAZ and SLC-0111 on the survival and development of living organisms and the changes in the oxidant/antioxidant system (MDA content, SOD and GST activity), which was experimentally induced by CCl_4, was performed.

2. Material and methods

2.1. The cultured organism

D. melanogaster (Diptera; Drosophilidae, W^{1118}) strain was bred for years in our laboratory (Necmettin Erbakan University Research Laboratories, Turkey). The larvae were kept in a standard fly medium containing mashed potatoes, agar, sucrose, dry yeast, ascorbic acid, and nipagin at 60 %–70 % humidity and a constant temperature of 25 ± 2 °C in darkness, as recommended by Lesch et al. [20].

2.2. Toxic trials

The CCl_4 used to generate toxicity was obtained from Sigma - Aldrich (St. Louis, MO). CCl_4 (5, 55, 125, and 195 mM) dissolved in 0.1 % dimethyl sulfoxide (DMSO) was added to standard fly medium, and the first-stage larvae obtained from the culture were transferred to this medium by means of a fine-tip brush. All experiments were conducted on three replicates, and 100 larvae were used in each replicate. After the application, the experimental setup was observed at 24, 48, 72, and 96-h intervals, the dead–live count was made, and the larval mortality rate was calculated. LC_{50} values were determined by Probit analysis (Probit Program Version 1.5; [21,22]).

2.3. Experiments against toxicity

The AAZ was purchased from Sigma-Aldrich, and SLC-0111 was synthesized and characterized as described in the literature by Nabih Lolak and Süleyman Akocak in Adiyaman University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology [8]. The stock solutions of AAZ and SLC-0111 were prepared by stirring with 0.1 % dimethyl sulfoxide (DMSO, Cas No: 67-68-5) for five minutes in a magnetic stirrer. In addition to 125 mM CCl_4 (LC_{50}), the experiment was set up with AAZ and SLC-0111 (2.5–10 mM) groups. For each experimental setup, 100 new and same-age larvae (first-stage) obtained from culture were inoculated into the experimental setup. Trials were performed in four replications.

2.4. Longevity experiments

The newly unmated flies were anaesthetized with CO_2 to distinguish between genders. The virgin females and males were retained for follow-up analyses. The adults (25–100 flies/replicate, 4 replicates/group) were, respectively, cultured and watched until death in experimental medium. The mediums were changed to fresh one every 3 days.

2.5. Biochemical assay

For each concentration, 20 larval, pupal, and adult individuals were washed in 20 % NaCl solution. Next, the fat tissues were removed under the dissection microscope. Samples were extracted in an ultrasonic homogenizer in a cold homogenization buffer (1.15 % potassium chloride, 25 mM dipotassium hydrogen phosphate, 5 mM ethylenediamine tetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol; pH: 7.4) at +4 °C. Total protein content was determined through the procedure described by Lowry et al. [23]. The Jain and Levine method [24] was used for the MDA quantity, Habig's method [25] was used for GST (EC 2.5.1.18) activity, and Marklund and Marklund's [26] method was used for the total SOD (EC 1.15.1.1) method. All chemicals were purchased from Merck (Darmstadt, Germany) and Sigma (St. Louis, Missouri, USA).

2.6. Data analysis

The statistics package program (SPSS Inc., Chicago, IL, USA) LSD Test to determine the significance of the difference between means analyzed the data. One-way analysis of variance (ANOVA; Tukey and Duncan's multiple comparison test) was used to compare data from control and application groups, and the chi-square test [27] was used to evaluate survival and sex ratio data. A value of $p < 0.05$ was selected for these analyses. The survival curves of the female and male populations were also drawn using Microsoft Windows Office Excel program. Results were represented as means ± standard error (M ± SE) for each experimental group in figures.

3. Results and discussion

The experimental setup for the current study was designed from the fact that cancer cell damage surrounding tissues and organs. Thus, the survival rate of the insects should be determined in relation to tissue damage and toxicity. When the larval mortality rates of the CCl_4 toxicity groups were examined, it was found that the toxicity increased linearly with increasing concentration and exposure time. After 96 h of administration of 125 mM of CCl_4, 50 % of individuals were killed and this dose of CCl_4 was determined to be LC_{50}. The larval mortality rate increased at the highest concentration (195 mM), with a mortality rate of 85 % ($p < 0.05$). Based on that, toxicity was created with 125 mM CCl_4, and AAZ and SLC-0111 were applied.

It was observed that increasing the amount of AAZ reduced the survival rate of larval individuals compared to the control group, whereas increased AAZ concentrations increased the survival to 27 %. However, it was determined that only SLC-0111-treated groups had a similar effect to the control group, and the survival rate was increased to 45 % (Fig. 1A). It was observed that SLC-0111 was more effective against toxicity than AAZ in the pupal and adult periods except the larval stage ($F_{30} = 2.92$, $p < 0.05$). However, the use of AAZ against CCl_4 in adulthood was not sufficient, and development reduced by about half (41 %–60 %).

However, the use of SLC-0111 prevented the toxicity generated during puberty process, and it was found that it increases survival rates by approximately 2.5 times compared to only CCl_4 (Fig. 1A; $F_{30} = 2.98$, $p < 0.01$). SOD activity limits or prolongs survival–development [28–30]. In this study, the fact that the substances used against CCl_4-induced toxicity increased survival rate is caused by increased SOD activity (Fig. 3C). Because increasing antioxidants increase the survival rate [31].

Organisms complete normal development processes over a longer period to maintain physiological balance and reduce the toxic effects of chemicals [32]. For example, the larval period of the fruit fly lasts three to four days, and the pupal period lasts about four to five days, while the adult period lasts approximately seven to eight days. The maturation process of larvae given CCl_4 increased to three days in larvae and 11 days in adults; in the groups treated with AAZ, the individuals reached the larval stage in six to seven days and reached puberty in about 14 days. In SLC-0111 treated groups, individuals became mature more quickly (eight to nine days), while with SLC-0111 and CCl_4 application, the duration of maturation increased to 13 days (Fig. 1B, $F_{30} = 2.16$, $p < 0.05$). Oral administration of AAZ to insect causes positive cytochemical reactions, but CA activity declines during the first 10 days of the adult *Drosophila* [33].

D. melanogaster males and females have different levels of DNA, RNA, and protein [34]. Therefore, males and females are affected differently from each other. For example, dyes affect men less [32]. With respect to the sex ratio, the use of CCl_4 increased male individuals while the using

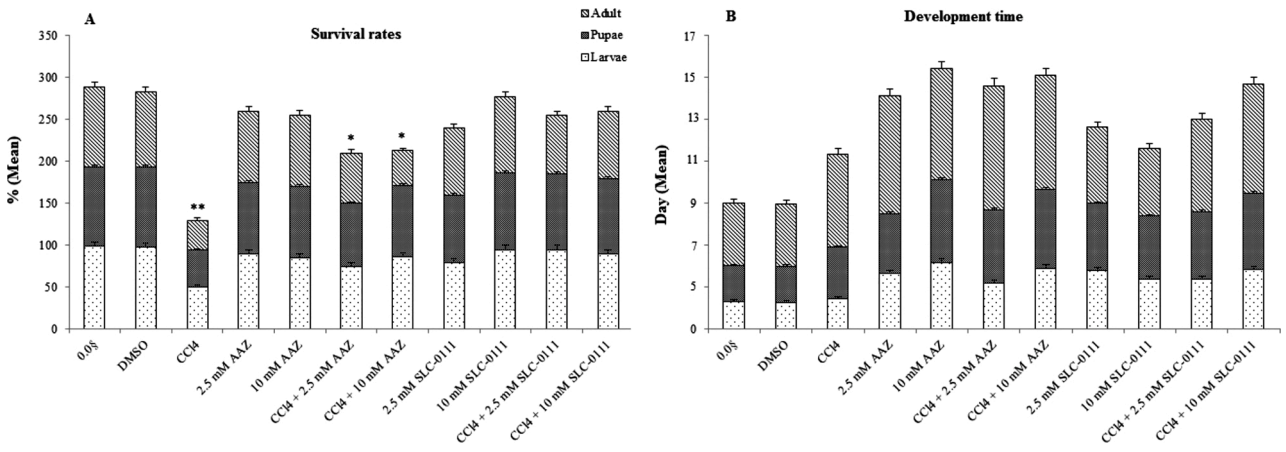

Fig. 1. The effects of acetazolamide (AAZ) and SLC-0111 on (A) survival rates and (B) development times of *D. melanogaster*. §Control medium (negative control), *p < 0.05, **p < 0.01, DMSO was used as a solvent, AAZ: Acetazolamide, CCl_4: carbon tetrachloride.

of AAZ increased female individuals. In SLC-0111 treated groups, it was observed that the sex ratios were similar to those observed in the control group (Fig. 2). Substances causing oxidative stress correlate negatively with sex ratio [35]. Additionally, in the CCl_4 and AAZ -treated groups, the abdominal region of the males was found to be small morphologically, whereas in CCl_4 and SLC-0111-treated groups, a morphological effect was observed in females.

CCl_4 has the most significant toxic effect on the liver and adipose tissue, which accelerates the formation of free radicals, and increases lipid peroxidation, thus showing the most significant toxic effect on liver and adipose tissue [36–39]. MDA, a substrate for ROTs, is the final product of lipid peroxidation resulting from the catabolization of unsaturated fatty acids [40]. In *D. melanogaster,* the fat tissue, which is equivalent to the human liver, exceeds the fat storage capacity, causing negative metabolic results [41]. Alternatively, the maximum amount of MDA in larval tissues was determined to be 10 mM SLC-0111 and 10 mM AAZ (Fig. 3A). Lipid peroxidation decreased in SLC-0111 and AAZ groups in pre-adult period, and CA reduces lipid biosynthesis [42]. Since SLC-0111 and AAZ are CA inhibitors, it is thought that the application of these substances alone decrease the amount of MDA in the insect. Although peroxidation with CCl_4 was reduced by feeding AAZ in pupal adipose tissue, it could not be reduced to less than 8.12 nmol/mg protein with the use of 10 mM SLC-0111. Simultaneously, 10-mM substances used against toxicity in adult individuals decreased the amount of MDA (Fig. 3A, $F_{30} = 2.16$, $p < 0.05$). The results of this study are in agreement with a previous study [43] that reported significantly increased GST activity, and first increased and then decreased concentration of MDA. In another study, a significant increase in lipid

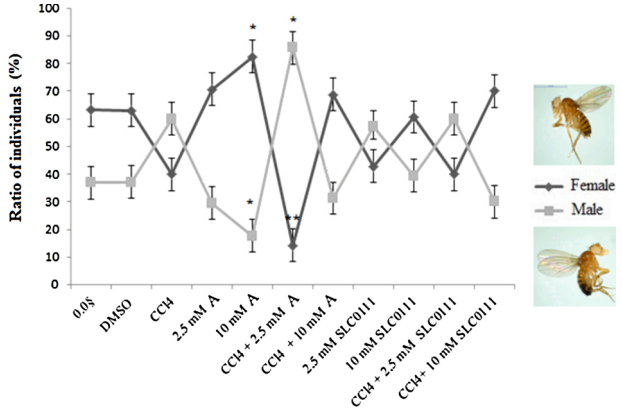

Fig. 2. The change in adult (male and female) ratios of *D. melanogaster* in the application groups.

peroxidation of *Setaria cervi* was observed with the use of high doses of AAZ [44]. Melatonin and quercetin were administered in rats despite tissue damage due to increased MDA by CCl_4. It was determined that CCl_4 increased MDA, and a decrease in GST and SOD activities was reported [37,45,46].

Many studies show that antioxidants can protect the body against CCI_4-induced oxidative stress [47–49]. The change in GST activity, which is an antioxidant enzyme, is also used as a marker of toxic effects. GST caused activity to be increased by adding 10 mM of the substances used in the larval stage to the food. SLC-0111 and AAZ concentrations in the pupal period were found to be resistant to the CCl_4 induced oxidative stress, whereas the use of AAZ and SLC-0111 during the maturation process increases the GST activity. The most notable increase in GST enzyme activity was seen because of continued feeding approximately 17 times as much CCl_4 and 10 mM AAZ compared to the control group (Fig. 3B, $F_{30} = 4.51$, $p < 0.01$). Compounds with low GST activity are preferred for treating cancer. For example, benzenesulfonamide used in *Ehrlich ascites* carcinoma cell lines may be preferred with low GST activity [50,51]. In adults, GST activity of the SLC-0111 is lower compared to the AAZ in toxicity groups (Fig. 3B).

In the developmental stages of the insect, 2.5 mM AAZ and SLC-0111 fed groups were found to have high SOD activities compared to the control group. It has been observed that the substances used against the CCl_4-induced toxicity in *D. melanogaster* increase the SOD activity and this increase is the highest in the pup and adult period (528 and 452 U/mg protein) with the use of 10 mM AAZ (Fig. 3C, $F_{30} = 4.51$, $p < 0.01$). The antioxidant enzymes SOD and GST activity were reported in a prior study to be increased in *Setaria cervi* treated with high doses of AAZ [44]. Thus, antioxidants against increased oxidation make the organism durable and increase its survival capabilities. In this study, it was seen that GST and SOD activity in adult tissues exposed to CCl_4 was higher than that of larval tissues. Our study supports the usability of AAZ and SLC-0111 due to their antioxidant properties. The AAZ and SLC-0111 increased the activity of antioxidant enzymes thus reducing the high percentage of survival in adults caused by the CCl_4 induced toxicity, extending life to approximately 49 days (Fig. 3D, $p < 0.05$). We observed that the maximum lifespan of the control group was 65 days; the maximum lifespan of the experimental group (AAZ) was 60 days and the maximum toxicity groups lifespan were 48 and 49 days, respectively. The difference between control, DMSO and only AAZ groups is not statistically significant (Fig. 3D, $p > 0.001$).

4. Conclusions

As a result, it was found that the use of AAZ and SLC-0111 were reduced CCl_4-induced oxidative stress in adipose tissue in

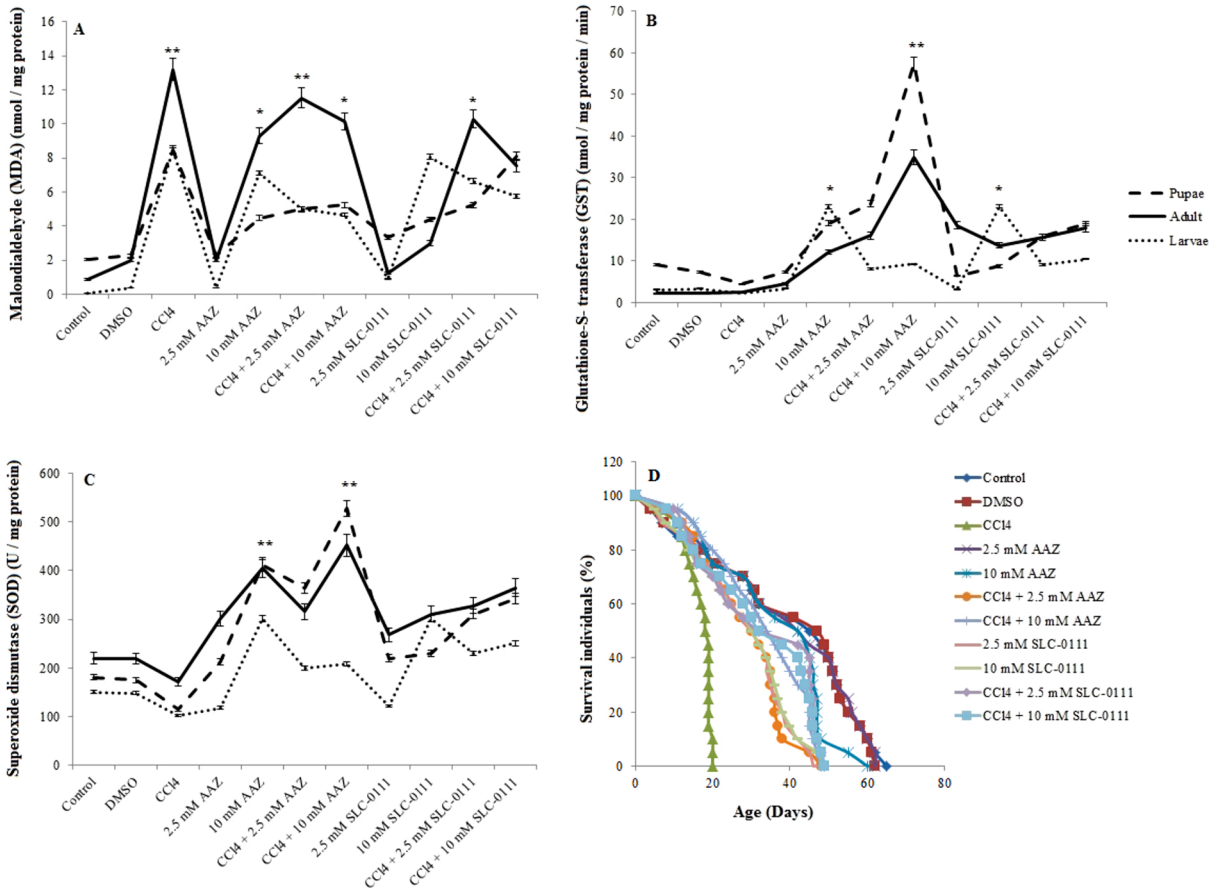

Fig. 3. Effect of AAZ and SLC-0111 of developmental stages of *D. melanogaster* treated with CCl_4 on (A) the amount of Malondialdehyde (MDA), (B) the activity of Glutathione-*S*-transferase (GST), (C) the activity of Superoxide dismutase (SOD) in adipose tissues and (D) the survival curves of the adults, *$p < 0.05$, **$p < 0.01$.

D. melanogaster. Thus, acetazolamide and SLC-0111 added to the food were positively affected the development of the insect. In terms of human health, it is important to conduct detailed in vivo and in vitro cytotoxicity and genotoxicity studies to understand the use of these substances against CCl_4. It is hoped that this study can shed light on new studies on reducing the harmful effects of CCl_4.

Authors' contributions

Conceptualization: E.G., H.A.; Methodology: E.G., H.F.N.; Validation: H.A.; Investigation: E.G., H.F.N.; Data curation: E.G., H.A.; Writing - original draft: E.G., H.A.; Writing - review & editing: E.G., H.A.; Visualization: E.G.; Supervision: H.F.N. All authors read and approved the final manuscript.

Funding

This research didn't receive grants from any funding agency in the public, commercial or not-for-profit sectors.

Conflict of interest

The authors declare no conflict of interest.

Declaration of Competing Interest

The authors report no declarations of interest.

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