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Paper

Involvement of functional senescence in efavirenz-induced toxicity in fruit fly

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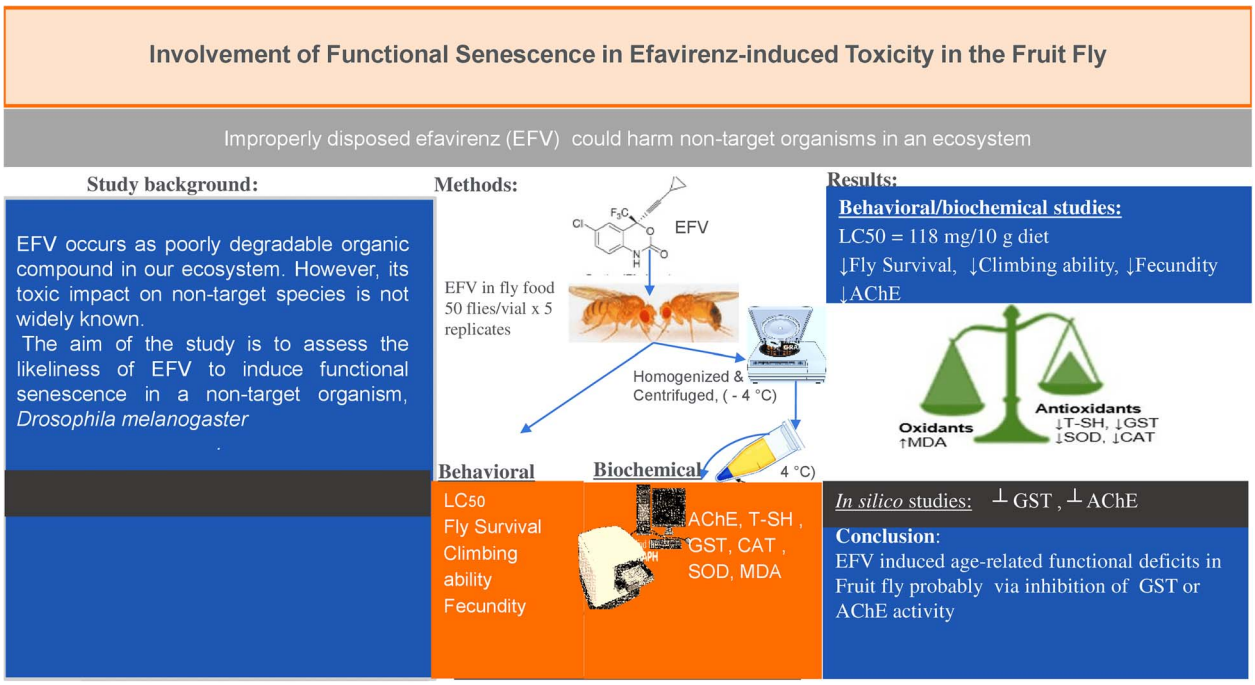
Background: We aimed in this article to assess the likeliness of efavirenz to induce functional senescence in *Drosophila melanogaster* (fruit fly).
Methods: Ten different concentrations of EFV were mixed with fly food and fed to 3-day-old flies orally for a 7 day LC_{50} calculation. Drug concentrations from LC_{50} were selected for a 28 day survival to determine the duration of treatment for behavioral and biochemical assays. A 5day feeding plan was used to investigate the effects of the drug on organismal, neuromuscular, reproductive, and metabolic senescence. An in silico study was executed to decipher a molecular interaction of Drosophila enzymes glutathione-s-transferase (GST) or acetylcholinesterase (AChE) with EFV.
Results: The calculated LC_{50} of EFV was 118 mg/10-g fly diet. The test drug induced a significant ($P < 0.05$) increase in fly mortality, climbing difficulty, and procreative deficits after a 5 day oral exposure. Similarly, there were significant ($P < 0.05$) biochemical alterations, which suggested *in vivo* biochemical damage against total thiols (T-SH), SOD (superoxide dismutase), CAT (catalase), GST, AChE, and MDA (malondialdehyde) in the test flies compared to the control groups. *In silico* study revealed a significantly ($P < 0.05$) higher binding energy between EFV and the active amino acids of fly AChE and GST when compared to the substrates or standard inhibitors respectively.
Conclusion: EFV exhibited ecotoxic potentials evidenced by age-related deficits in the fly's functional integrity such as sluggish movement, procreative deficiency, increased mortality, and oxidant-antioxidant inequality. Results from *in silico* study suggested antagonism against GST and AChE activities as a likely mechanism of EFV-induced toxicity in the fruit fly.

Graphical Abstract



Key words: functional senescence; efavirenz; Drosophila melanogaster; oxidative stress; acetylcholinesterase; glutathione-S-transferase.

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Introduction

In 2021, an estimated 38.4 million [33.9–43.8 million] people were infected with the human immunodeficiency virus (HIV-1) globally, with 28.7 million people currently on antiretroviral drugs.[1] Two-thirds (25.6 million) of the world's HIV-1 burden and people on antiretroviral drugs are from African countries.[1,2] Efavirenz is no longer a mandatory component of first-line highly active antiretroviral therapy (HAART).[3] Nevertheless, it is still a recommended alternative at a low dose (400 mg) in combination with two other drugs (lamivudine and tenofovir disoproxil fumarate or emtricitabine) for female patients desiring pregnancy or those without access to reliable birth control.[4,5] Illicitly, efavirenz tablets may be powdered and smoked to achieve a state of ecstasy.[6] Therefore, efavirenz may be deposited into the environment through poor disposal of the pharmaceutical product, which may continue to affect different species of life in an ecosystem.[7]

Aside from poor disposal practices, the environment may become contaminated with antiretroviral drugs through untreated sewages (urine and feces)[2] as well as wastewater from drug manufacturing facilities or hospital effluents used for irrigational purposes.[8] There are empirical pieces of evidence that efavirenz together with its major metabolite 8-hydroxy efavirenz are poorly degradable,[2,9,10] thus its persistence in the environment could be hazardous to human and non-target species as well.[10] In Africa, antiretroviral drugs have been detected above safety limits in surface waters,[2] agricultural soils, and plant tissues.[8,11] This is worrisome because many rural communities depend on surface waters for consumption,[2] and agricultural practices.[11] In a natural habitat, *Drosophila melanogaster* feeds on ripped and rotting fruits, moist organic matter, and dead insects via oral route. Plants may take up poorly disposed efavirenz in the environment and accumulate it in their tissues (including ripped or rotting fruits), or the drug residue may directly contaminate sources of fly food in the natural food chain.[7] Therefore, understanding the ecological effects of efavirenz-induced toxicity on lower organisms is an essential proponent for the development of remediation plans.

Efavirenz induces adverse physiological effects resulting in alterations in human system functions at therapeutic doses.[12,13] After oral exposure, EFV is quickly absorbed into the systemic circulation and reaches maximum plasma concentrations within 3 to 6 h.[14] It is then metabolized by the cytochrome P450 enzyme (CYP2B6) to an active neurotoxic metabolite 8-hydroxy-EFV (8OH-EFV). 8HO-EFV causes cytotoxicity by inducing mitochondrial function deficits, and activation of oxidative stress signaling pathways.[13,15] Oxidative stress is characterized by excessive production of free radicals, also known as reactive oxygen and nitrogen species (RONS) *in vivo*, leading to the death of cells, tissues, and ultimately the organism.[16,17] RONS are known culprits in the pathogenesis of several human diseases including diabetes mellitus, neurodegenerative diseases, cancer, and physiological aging.[18,19] Additionally, several reports have linked oxidative stress to reproductive deficits in both rodents[20] and humans.[21] Although efavirenz-induced oxidative stress is well documented in human[12,13,22] and animal models,[20,23] its ecotoxicological effect on insects particularly *D. melanogaster* is scarcely available.

The fruit fly is a good model organism in toxicology[24] because of its high (77%) genomic similarity to several genes implicated in human diseases.[25] It is a powerful tool for investigating the physiology of age-related functional senescence, which interrupts the quality and lifespan of an organism.[26] The enzymatic antioxidant system in vertebrates is also conserved in *D. melanogaster*.[24] Exposure of *D. melanogaster* to toxicants could be achieved through ingestion, injection, or inhalation.[24,27] Nevertheless, the oral route is the most convenient route of exposure to efavirenz in its solid dosage form in flies. Thus, the feeding method was employed to investigate the ecotoxicological potential of EFV in a Harwich strain of fruit fly in the current study.

Materials and methods

Reagents and antiretroviral drug

Efavirenz 600 mg/tablet (Cipla Ltd) was donated by General Hospital Gboko, Benue State, Nigeria. Ten (10) tablets of the test drug were weighed on an analytical balance (Meltlar–MT200B), and the average weight was calculated, then pulverization in a porcelain mortar and pestle. The amount of active EFV needed was calculated relative to the excipients and weighed out. To enhance the solubility of EFV, a stock concentration of 40 mg/mL was prepared with no observed adverse effect concentration of 0.3% dimethyl sulfoxide (DMSO).[28] Aliquot EFV concentrations of 30 mg, 15 mg, 7.5 mg, and 3.75 mg per 1,000 μL were obtained via dilution with distilled water and added to cold fly food to yield a 10 g mixture. Two sets of controls (with 0.3% DMSO or distilled water) were used in this study but only the DMSO 0.3% data result is shown hence there was no statistical difference between the two. The highest concentration of EFV used in the current investigation was 25% of the calculated LC_{50} based on observed mortality rates from our pilot studies (result not shown).

The chemical reagents used for this study were of analytical grade. Hydrogen peroxide (H_2O_2), GSH, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and 1-chloro-2,4-dinitrobenzene (CDNB was from the Drosophila laboratory, Africa Centre of Excellence in Phytomedicine Research and Development (ACEPRD), university of Jos. 30% Trichloroacetic acid, 0.75%, Thiobarbituric acid, Tris-KCl buffer (0.15 M, pH 7.4), carbonate buffer (0.05 M, pH 10.2), 0.1 M HCl, and 0.3 M Adrenaline were purchased from the Department of Biochemistry, National Veterinary Research Institute, Vom, Jos Nigeria.

Fruit fly culture

The Harwich strain of fruit fly was fed on yellow corn medium composed of 1% w/v agar, 2% w/v brewer's yeast, and 0.08% w/v methylparaben as a preservative. The flies were housed under approved relative humidity (60%), temperature (23 ± 1 °C), and 12 h dark/light cycles[29] at ACEPRD University of Jos.

Evaluation of median lethal concentration (LC_{50}) and survival assay

The median lethal concentration (LC_{50}) of EFV in fruit flies was evaluated using a published protocol.[30] LC_{50} was evaluated to guide the choice of experimental concentrations that could show biochemical and organismal toxicity with mortality below 30%. Five clusters of sixty (60) flies in five replicates each were exposed to ten (10) varying concentrations of the experimental drug via feeding method for 168 h. Fly deaths were recorded every 24 h throughout the experimental period and the mortality rate was subjected to a dose-response simulation in GraphPad Prism 8.0.2 software to calculate LC_{50}. Efavirenz concentrations equivalent to 25% of the calculated LC_{50} or below were used in the present investigation due to the observed fly lethargy above this limit in our pilot studies.

The survival rate of experimental flies exposed to EFV was evaluated using the method of Abolaji et al.[29] The survival of

EFV-exposed flies was evaluated because increased functional senescence could present as accelerated aging and premature deaths. Environmental conditions suitable for fly research were maintained as described by Abolaji et al.[29] throughout the investigation. Dead flies were counted and recorded every 24 h for 28 days. Thereafter, percentage mortality was calculated and plotted against time (days) on a Kaplan-Meier curve. The curve was used to determine the appropriate duration of treatment for climbing performance, hatching ability, and biochemical evaluations before flies could enter a death rate above 30%.

Fly treatment for climbing ability, reproductive capacity, and biochemical assays

A 5day duration of fly exposure to EFV was read-off on the 28 day Kaplan-Meier curve corresponding to 70% survival (Fig. 2A). The 5th-day was chosen as the duration of fly exposure to EFV because signs of EFV-induced toxicity among the exposed groups, (e.g. increased deaths and sluggish fly movement),[31] were noticeable. After a 5 day fly exposure to EFV, the published protocol by Adedara et al.[32] was used to assess the ability of the exposed flies to climb against gravity. Negative geotaxis (escape ability against gravity) is a behavioral assay used to evaluate neuro-muscular senescence in fruit flies.[26,31] The ability of adult *Drosophila melanogaster* to copulate, lay eggs, and hatch following a 5 day exposure to EFV was investigated as described by Pam et al.[33] The total number of flies that were able to hatch during the experimental period was inferred as the measure of the reproductive ability of the EFV-exposed flies. Decreased procreation under oxidative stress conditions implies reproductive senescence in fruit flies,[34] which was observed previously among humans on antiretroviral drugs.[35] Therefore, the reproductive ability of EFV-exposed flies was investigated to find out if the test drug could induce fertility deficits in insects.

For biochemical studies, flies previously exposed to EFV through the feeding method for 5 days were subjected to cold shock (by placing vials containing the flies under ice), then weighed and homogenized in 0.1 M phosphate buffer (pH 7.4) using a flyweight to buffer ratio of 1 mg: 10 μL. Eppendorf cold centrifuge (model: AG, 5227 R) was set at 4,000 revolutions per 600 s at a temperature of −4 °C to spin the whole fly homogenate. The supernatant was collected using a micropipette into clean Eppendorf tubes for the evaluation of total protein, total thiols, and malondialdehyde levels; as well as the activities of some enzymes like catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), and acetylcholinesterase (AChE).

Evaluation of biochemical parameters

Total protein content was assayed and used in this investigation to adjust other biochemical parameters and expressed results as fractions of fly protein. Also, some enzymatic (CAT, SOD, and GST) as well as non-enzymatic (T-SH) antioxidants were evaluated because both EFV and 8HO-EFV are pro-oxidants,[13,15] thus the fly antioxidants system could be susceptible. In addition, both increase or decrease in AChE activity could affect fly health adversely and may predict a propensity for oxidative stress, neurological toxicity, or deficits in muscular tone in fruit flies.[21] Thus, AChE might be a target enzyme for EFV-induced toxicity. Finally, the MDA level was quantified and used as a measure of free radicals circulating in *D. melanogaster* and was useful in predicting the overall oxidant/antioxidant balance in the model organism.

The Bradford method described by Omale et al.[36] was adopted to evaluate protein content in the fly homogenate and expressed as total protein per milligram. For Total thiol evaluation, Ellman's method modified by Abolaji et al.[27] was utilized. The reaction medium and all the prescribed experimental conditions were maintained as described by Abolaji et al.[27] Total thiol content was determined from a standard glutathione curve and expressed as mmol/mg protein. GST activity was determined using the Habig and Jakoby's method.[27] An extinction coefficient (ε) = 9.6 mM^{-1} cm^{-1} was used to quantify GST activity and expressed in nmol/min/mg protein. Ellman's method[37] was implemented to determine the activity of AChE. A medium for the reaction contained 285 μL of distilled water, 180 μL of 100 mM potassium phosphate buffer (pH 7.4), 60 μL of 10 mM DTNB, and 15 μL of fly homogenate. 60 μL of 8 mM acetylthiocholine was added last to start the chemical reaction. The wavelength of a spectrophotometer (Jenway number 7315) was set at 412 nm, to run for 120 s at 10 s intervals. AChE activity was calculated and expressed in nanomole (nmol)/min/mg protein.

Superoxide (SOD) activity was evaluated using the method of Misra and Fridovich, as modified by Iorjiim et al.[30] The content of the reaction medium and other experimental conditions and settings were maintained as described by Iorjiim et al.[30] SOD was calculated and expressed in micromole (μmol)/min/mg protein. Catalase activity was investigated using Aebi's modified by Abolaji et al.[27] A stock solution comprising 100 mL of potassium phosphate buffer (pH 7.0) plus 194 mL of 19 mM hydrogen peroxide (H_2O_2). Exactly 590 μL of the stock solution was added to (10 μL) of fly homogenate and the rate of H_2O_2 consumption was determined under room temperature (25 °C) at a wavelength of 240 nm, and result expressed in micromole (μmol) of H_2O_2 consumed/min/mg protein. Malondialdehyde level was evaluated as a measure of *in vivo* RONS generation in the fly homogenate following the method of Varshney and Kale described by Iorjiim et al.[30] The calculated MDA level was quantified in micromole (μmol)/mg protein.

Modeling of molecular interactions *in silico*

The nature of the molecular interaction of EFV and Drosophila AChE and GST enzyme was investigated using molecular docking.

Identification and collection of ligand and protein

The structure-data file (SDF) formats of the required chemical structures for *in silico* modeling such as EFV (ID: 64139), 6-[(7-nitro-2,1,3-benzoxadiazol- 4-yl)sulfanyl]hexan-1-ol, (NBDHEX, ID: 9817686), glutathione (ID: 124886 ID), galantamine (ID: 9651), and acetylcholine (ID: 187), were identified from literature[38,39] and obtained from Pub-Chem database in their 3D formats. Also, the appropriate crystallographic structure of *Drosophila melanogaster* GST (protein data bank ID: 1 M0U) and AChE (protein data bank ID: 1Q0N) were appropriately identified,[38,40] then downloaded from the protein data bank.

Ligand and protein preparation for molecular docking

For ligand and protein preparation for docking, a method described by Johnson et al.[40] was used. A file containing previously identified ligands was imported, minimized, and converted to their appropriate auto-dock ligand formats (PDBQT) using PyRx software.[40] With the aid of appropriate protein IDs, AChE, and GST were retrieved from the protein data bank into the UCSF Chimera (version 1.14), made suitable for docking as described by Johnson et al.[40] and stored in PDB format.

Ligand-protein complex formation

The AChE and GST structures previously stored in their respective PDB formats were loaded to PyRx software and designated as macromolecules then converted to PDBQT formats. Each

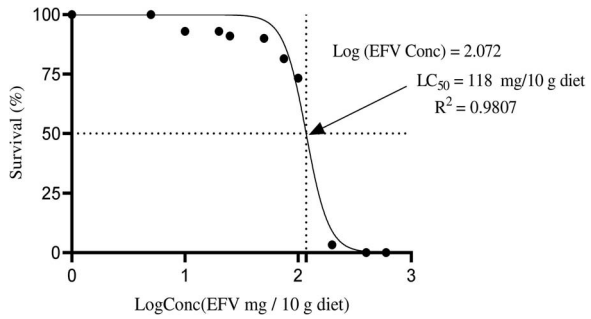

Fig. 1. LC_{50} of efavirenz after a 7 day exposure through feeding method in the fruit fly. Data are presented as mean ± SEM of five independent replicates.

macromolecule was made to interact with its respective standard inhibitor, natural substrate, and EFV while maintaining the grid box at the software's default. Outputs of the docked results were exported as zip files to PyMol (version 2.3.3) for visualization and analysis. The generated 2D and 3D models of the possible molecular interaction were finally viewed using Discovery Studio (version 2020) software.

Statistical analysis

Data were presented in the form of Mean ± SEM in GraphPad Prism version 8.0.2 for Windows. The statistical tool for analysis was one way ANOVA (analysis of variance) with Tukey's post hoc test to distinguish means with significant statistical differences at $P < 0.05$. Analysis of survival curves was performed with Log-rank (Mentel-cox) test using a Bonferroni adjusted P-value/K, where $K = 4$ comparisons. Thus, curves were considered statistically significant at $P < 0.01$).

Results

168 h (7 day) LC_{50} determination

Exposure of *Drosophila melanogaster* to EFV for 7 days showed an LC_{50} = 118 mg per 10 g fly food (Fig. 1). Severe fly lethargy was observed in 80% of the surviving flies from experimental day 6 in groups fed with a 30 mg per 10 g diet (25% LC_{50}) and above (result not shown).

28 day survival rate of EFV-exposed flies

Exposure of fruit flies to EFV through the oral route significantly ($P < 0.05$) accelerated organismal senescence observed as decreased survival at all experimental concentrations compared to the unexposed groups. The increased fly mortality was directly proportional to EFV concentrations (Fig. 2A and B).

Five day mortality rate, climbing ability, and fly emergence (procreative capacity)

The result of 5 day fly exposure to Efavirenz significantly ($P < 0.05$) increased organismal senescence (m-aortality rate), motor senescence (decreased climbing), and reproductive senescence (declined hatching ability) respectively (Fig. 3A–C).

Changes in biochemical parameters of EFV-exposed flies

After a 5 day exposure (Fig. 4A–F), Efavirenz-induced metabolic senescence in *D. melanogaster* was observed as significant ($P < 0.05$) reductions in the level of T-SH as well as activities of CAT and AChE at all experimental concentrations compared to the unexposed. Similarly, there was a significant ($P < 0.05$) decrease in the activities of SOD at 15–30 mg/10 g diet, GST at 7.5 mg–30 mg per 10 g diet with an associated significant ($P < 0.05$) increase in MDA concentration at 15 mg–30 mg per 10 g diet when compared to unexposed flies.

In silico Modeling of molecular interactions

The result of in silico molecular interactions is as shown (Fig. 5A and B). EFV exhibited a significantly ($P < 0.05$) stronger binding force for GST and AChE amino acids compared to the respective binding energies exhibited by inhibitors (galantamine and NBDHEX) and the natural substrates (acetylcholine and glutathione).

Ligand-protein interactions

Efavirenz interacted with some amino acids such as TYR A: 54, ARG A: 145, and TYR A: 208 at GST binding surface similar to glutathione and NBDHEX (Fig. 6A–C). At the AChE enzyme (Fig. 7A–C), EFV, galantamine, and acetylcholine competed for the same active amino acids residues such as TRP A: 83, TYR A: 370, and HIS A:480. In addition, both AChE inhibitor (galantamine) and EFV interacted with another amino acid residue TRP A: 71.

Discussion

Functional senescence is a steadily decreased cellular, organ, system, or organismal function as a result of aging, illness, or exposure to environmental toxins.[26,40] The current study has shown that Drosophila's functional ability declined steadily, which was attributable to EFV-induced toxicity after oral ingestion. The EFV-induced organismal senescence was observed as significantly ($P < 0.05$) increased fly mortality from 13. 7% (control) to 80.7% (30 mg/10 g diet). Other indices of senescence observed among the experimental flies were neuro-muscular, reproductive, and antioxidant defense deficits. These efavirenz-induced toxicities in this study were previously reported as aging phenotypes, which correlated positively with oxidative stress in *D. melanogaster*.[34] Several studies have shown that chronic oral administration of efavirenz-based HAART by HIV patients induced muscle weakness,[41] reproductive decline,[42] increased mortality rate, and oxidant-antioxidant imbalance compared to the uninfected population not exposed to antiretroviral drugs.[35,43,44] The results of the lethal median concentration (Fig. 1) together with 28th day survival (Fig. 2A–B) and 5 day mortality assays (Fig. 3A) in this study confirmed that EFV was toxic to the exposed flies compared to unexposed groups. The calculated LC_{50} of efavirenz in the current study may not correlate perfectly with the environmental dosages. This is because environmental concentration of efavirenz is dynamic and may increase over time due to the poor degradable and bio-accumulative nature of the drug. EFV induces mitochondrial dysfunction in humans, resulting in decreased cellular respiration, increased inflammatory signals, and the production of free radicals.[13,45,46] Therefore, the significant ($P < 0.05$) increase in deaths among the EFV-exposed flies when compared to the controls in the current study could be attributed to efavirenz-induced functional deficits of the antioxidant system.

EFV significantly ($P < 0.05$) reduced climbing, performance, hatching ability (Fig. 3B and C), and acetylcholinesterase activity (Fig. 4E) in fruit flies after a 5 day oral exposure. Acetylcholine (ACh) is a neurotransmitter that mediates brief neurochemical signal control at neuromuscular junction and gland cells, after which its action is terminated by the AChE enzyme.[47] Thus, a significant reduction in AChE activity favors neuronal ACh

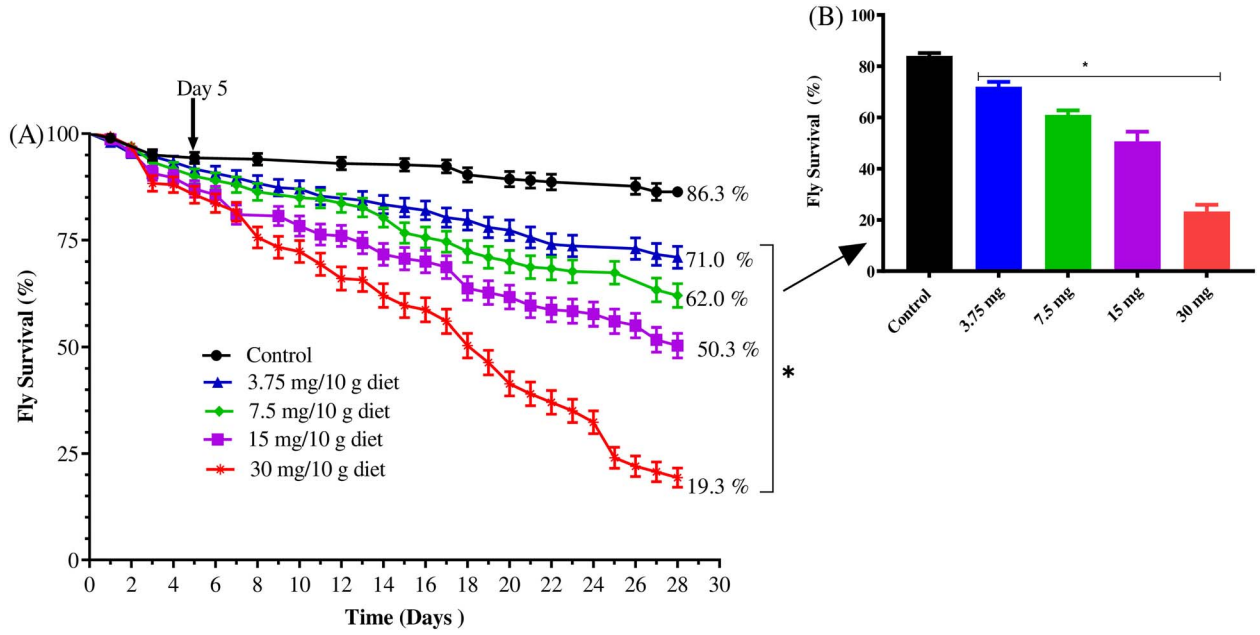

Fig. 2. A, B) EFV significantly decreased the survival rate of fruit fly dose-dependently after 28 day exposure through oral route. A) Daily survival proportions, B) 28th day survival rate. *Significantly ($P < 0.05$) lower compared to unexposed group. Data are presented as mean ± SEM of five independent replicates.

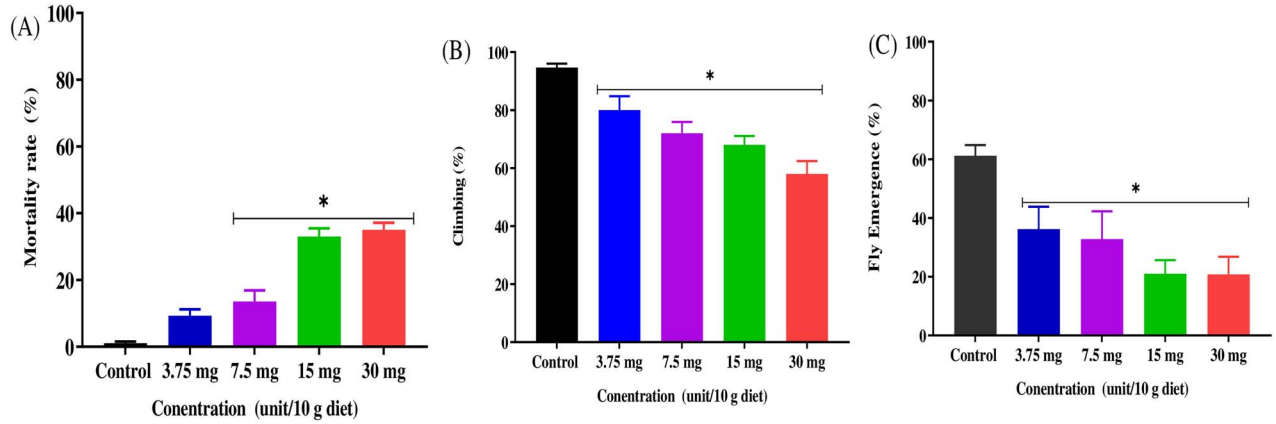

Fig. 3. A–C). Effects of EFV on A) mortality rate, C) climbing ability (negative geotaxis), and C) Emergence rate in fruit fly after a 5 day exposure. *Significantly ($P < 0.05$) different compared to the unexposed group. Data are presented as mean ± SEM of five independent replicates.

accumulation causing muscle paralysis due to sustained action potential.[47] Our results agreed with Haghnazari et al. who reported that insecticides containing AChE inhibitors impaired the quantity and quality of fly sperms.[21] Alterations in the function of the cholinergic system have been implicated in human and other vertebrate models of neurodegenerative diseases,[48] however, the particular consequence of cholinergic deficits in fruit flies is not fully elucidated.[27] Nevertheless, either an increase or decrease in AChE activity has been shown to impact the health status of *D. melanogaster* negatively.[27] Our current findings disagreed with Urra et al.[49] who reported enhanced fertility in rats following AChE inhibition with Huperzine A. A possible explanation could be that Huperzine A is an antioxidant molecule from the *Huperzia serrata* plant,[50] which could ameliorate oxidative stress-induced infertility.[51] Therefore, EFV might have reduced the fly fertility and climbing ability through its inhibitory effect on the fly AChE activity.

The biological aging of metabolic processes in fruit flies can be assessed by evaluating their antioxidant system competence.[26] Therefore, some biomarkers of oxidative stress in EFV-exposed flies were investigated to further understand the impact of the test drug on fly senescence. Moderate oxidative stress may initiate the production and release of the antioxidant response element (ARE) gene, which is responsible for the synthesis of antioxidant enzymes such as catalase.[52] However, excessive ROS production above the antioxidant capacity induces oxidative injury to DNA, proteins, and lipids, resulting in loss of organismal functions and ultimately death.[53] EFV in the current study decreased all the evaluated antioxidant biomarkers (T-SH, CAT, SOD, and GST), and concomitantly increased MDA contents in vivo (Fig. 4A–F), implying an imminent state of EFV-induced oxidative stress in *D. melanogaster*. Malondialdehyde is a toxic byproduct of ROS-induced lipid peroxidation used as an indicator of circulating free radicals.[54] In our previous investigations, antiretroviral drugs lamivudine, tenofovir disoproxil fumarate,[55] or dolutegravir[56] significantly elevated MDA levels in *D. melanogaster* following oral exposures. Similarly, the result of MDA in the current study agreed with Adaramoye et al. who observed a significant increase in MDA

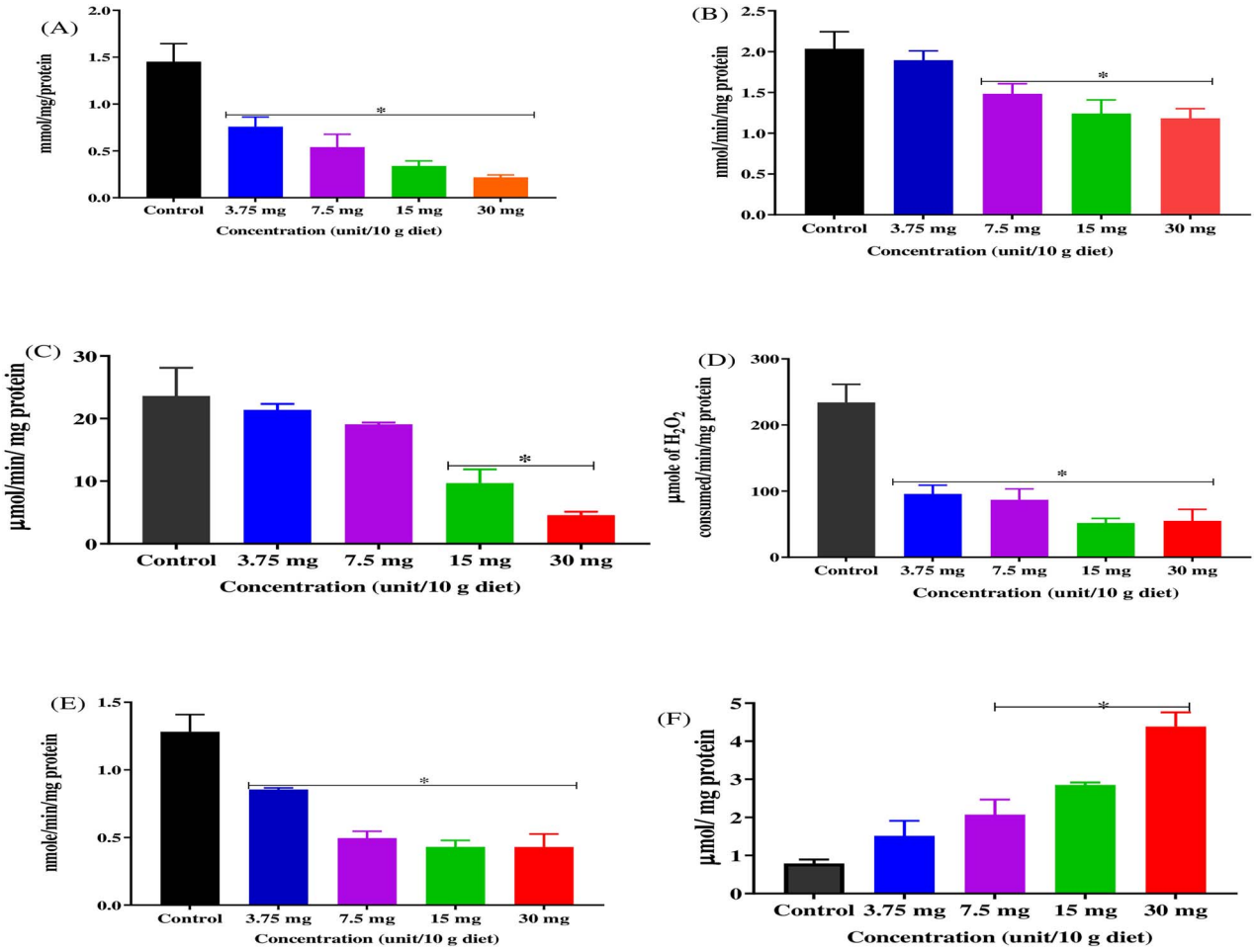

Fig. 4. A–F) Changes in biochemical parameters after 5 day exposure of fruit fly to EFV. *Significant ($P < 0.05$) difference compared to unexposed flies. Data are presented as mean ± SEM of five independent replicates. A) Total thiols, B) Glutathione-S- transferase, C) Superoxide dismutase, D) Catalase, E) Acetylcholinesterase, and F) Malondialdehyde.

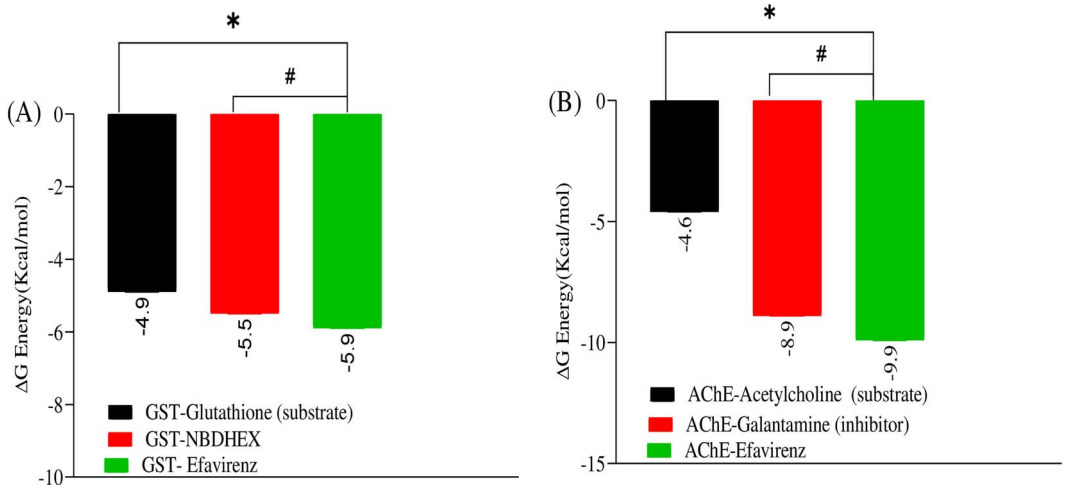

Fig. 5. A, B) Binding energies of fly enzyme-ligand complexes. A) GST-EFV, GST-NBDHEX, and GST-glutathione binding energies. B) AChE-EFV, AChE-Galantamine, and AChE-acetylcholine binding energies. *EFV-enzyme affinity compared to the substrate-enzyme complex is significantly ($P < 0.05$) different, #EFV-enzyme affinity compared to the inhibitor-enzyme complex is significantly ($P < 0.05$) different. FV = efavirenz, GST = glutathione-S-transferase, AChE = acetylcholinesterase, NBDHEX = 6-[(7-nitro-2,1,3-benzoxadiazol- 4-yl) sulfanyl]hexan-1-ol.

levels after 28 days of exposure to antiretroviral drugs Nevirapine[57] or Tenofovir[58] in rodents.

The assayed biochemical parameters in the current study (T–SH GST, SOD, and CAT) (Fig. 4A–E) are essential parts of the antioxidant system that defends fruit flies against the injurious effects of ROS *in vivo*.[59] For example, thiols (e.g. homocysteine (HcySH and glutathione (GSH) possess sulfhydryl groups (-SH), which preferentially react with free radicals or xenobiotics.[59]

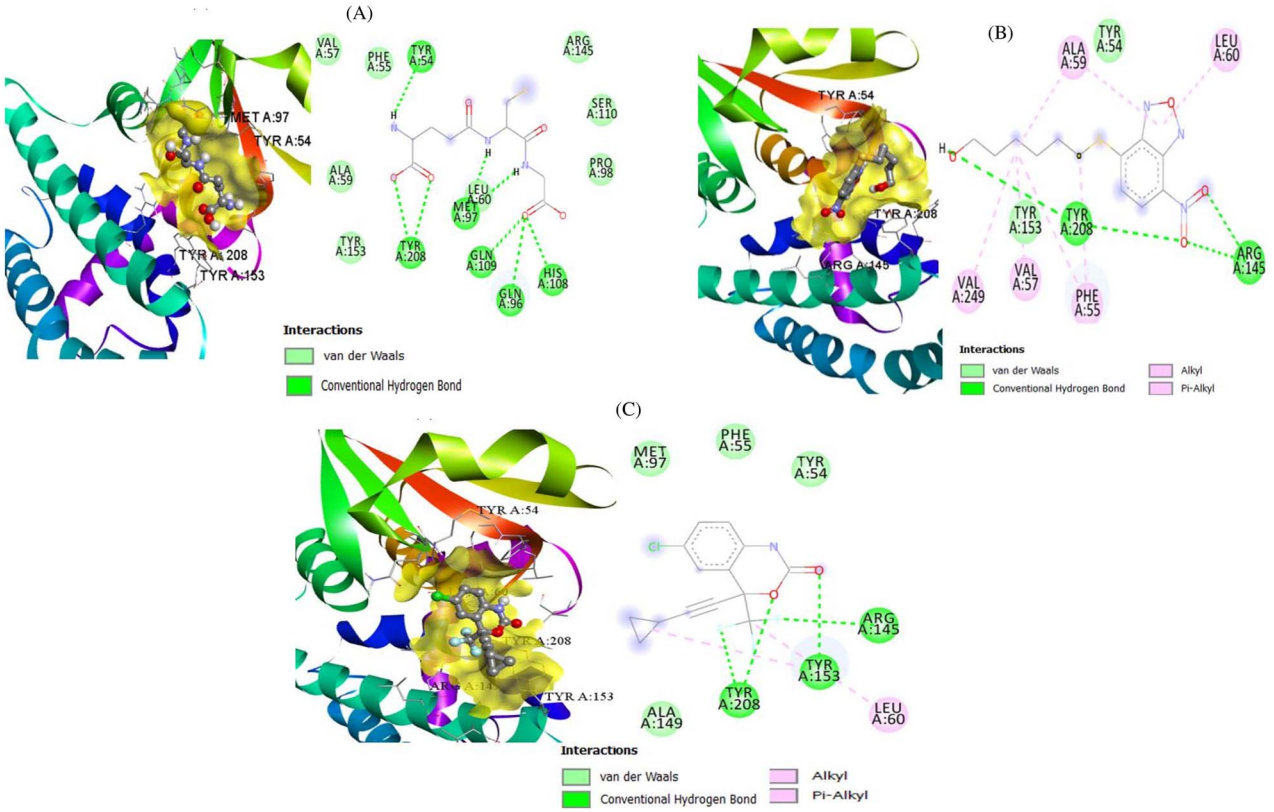

Fig. 6. A–C) Right (2D) and left (3D), presentation of the molecular interactions between the amino acid residues within GST binding pocket and GSH (a substrate), NBDHEX (a standard antagonist), and efavirenz (EFV). A) Glutathione-S-transferase-glutathione complex, B) Glutathione-S-transferase-NBDHEX complex, and C) Glutathione-S-transferase- Efavirenz complex.

Glutathione-S-transferases possess catalytic activity, which catalyzes the conjugation reactions between thiols and electrophiles.[60] Relatedly, catalase and superoxide dismutase protects living cells from free radical injury.[61] First, superoxide dismutase is involved in the conversion of two molecules of superoxide anions ($2O_2^{\bullet-}$) to one molecule of hydrogen peroxide (H_2O_2) and an oxygen molecule (O_2). Second, catalase converts the H_2O_2 from the first step into O_2 and two water molecules.[61] Hydrogen peroxide is a non-radical reactive molecule, which produces more reactive species such as hydroxyl radical (HO•) or ferryl ion $[Fe(IV) = O]^{2+}$ upon interaction with ferrous ion (Fe^{2+}) in the Fenton reaction.[62] Therefore, the significant decrease in these antioxidant molecules with a corresponding significant MDA elevation after EFV exposure in the current study might have impeded the metabolic functional integrity of the fly's antioxidant system. This result was consistent with other reports of antiretroviral-induced toxicities in rat models[42,58] and *D. melanogaster.*[55,56,63]

An *in silico* study to decipher a probable mechanism of EFV toxicity in *Drosophila melanogaster* was performed against some active amino acid residues of *D. melanogaster* enzymes acetylcholinesterase and glutathione-S- transferase.[64] These enzymes were selected for molecular docking because of their susceptibility to EFV-induced toxicity and the accessibility of their 3D structures at the protein data bank. In this study, the results of EFV exposure against behavioral and biochemical effects in *D. melanogaster* were consistent with that of *in silico* interaction between the drug and some susceptible enzymes (GST and AChE). EFV showed a significantly ($P < 0.05$) stronger attractive force for AChE and GST than the respective substrates and standard inhibitors (Fig. 5A and B). At the GST binding pocket (Fig. 6A–C), EFV interacted with catalytic amino acid residues such as TYR A: 54, ARG A: 145, and TYR A: 208.[65] Also, EFV interacted with catalytic amino acids of AChE: TRP A: 83, TYR A: 370, and HIS A:480[66,67] (Fig. 7A–C). This current result is consistent with our previous study, which revealed a potentially toxic interaction between these drosophila enzymes through the same active sites with Lamivudine and Tenofovir disoproxil fumarate.[55] In another study, *in silico* inhibition of Drosophila AChE and GST by Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide correlated positively with its *in vivo* antioxidant functions.[40] Therefore, the current *in vivo* and *in silico* investigations jointly suggested a competitive antagonism of efavirenz against glutathione-S-transferase and acetylcholinesterase as a likely mode of EFV-induced toxicity in fruit flies.

Conclusion

Oral administration of EFV to fruit flies resulted in a significantly induced senescence of metabolic function observed as decreased antioxidant biomarkers and increased free radical generation. The EFV-induced oxidative stress toxicity may have contributed to the observed age-related organismal functional deficits namely, climbing deficits, reproductive decline, and increased mortality rate in the current study. The result of the *in silico* study suggested the involvement of competitive antagonism against acetylcholinesterase and glutathione-S-transferase enzymes as a mode of EFV-induced functional senescence in fruit flies. Thus, environmental pollution with EFV could threaten the existence of *Drosophila melanogaster* in an ecosystem.

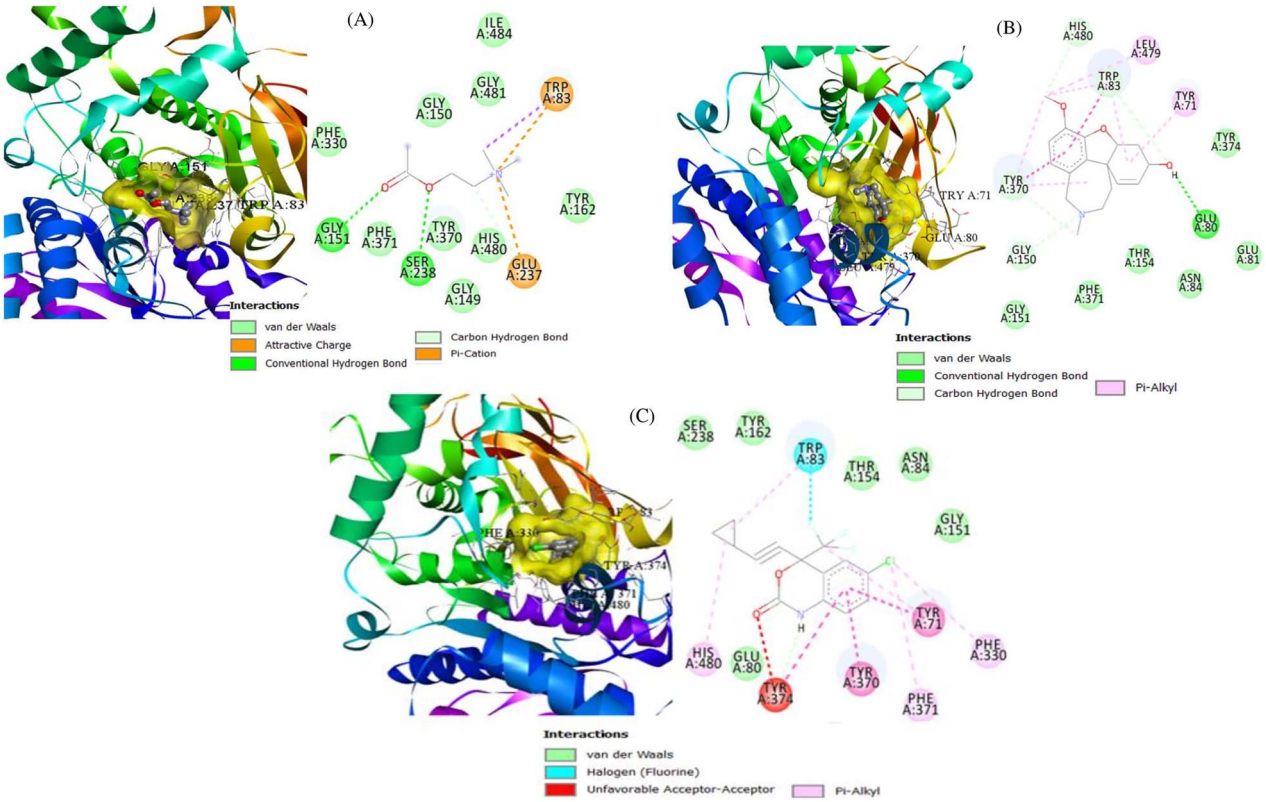

Fig. 7. A–C) Right (2D) and left (3D), presentation of the molecular interactions between the amino acid within the acetylcholinesterase (AChE) binding pocket and acetylcholine (natural substrate), NBDHEX (standard antagonist), and efavirenz (EFV). A) AChE—Acetylcholine, B) AChE—Galantamine complex, and C) AChE—Efavirenz complex.

Author contributions

WMI conceived and designed the study. WMI and CVU managed the literature searches. All authors contributed in conduction of the experiments and collection of the data. WMI handled the analysis of the data and wrote the first draft of the manuscript. All authors approved the final manuscript.

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Data availability statement

All relevant data are within the articleand its Supporting Information files.

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