

The association of cysteine to thiomersal attenuates its apoptosis-mediated cytotoxicity in zebrafish

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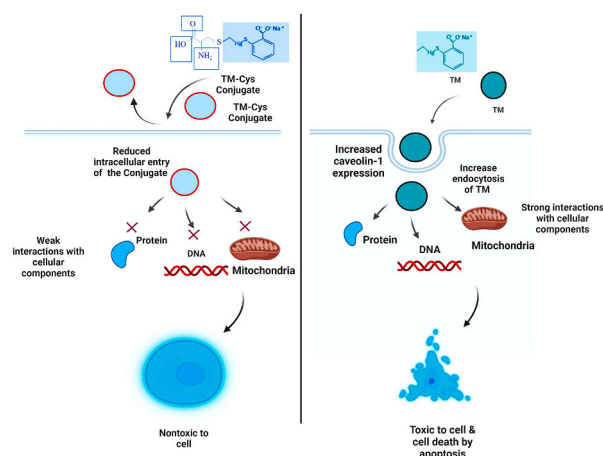
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HIGHLIGHTS

- TM causes apoptosis by interacting with intracellular proteins and mitochondria.
- Equimolar mixture of cysteine and TM stops TM-mediated toxicity.
- Cysteine forms a stable conjugate with TM.
- Conjugated form of TM still possesses a decent bactericidal efficacy.
- Cysteine combination is the safer alternative to nullify the TM-mediated toxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Thiomersal (TM) is an excellent preservative that is used in a wide variety of products, like pharmaceuticals, cosmetics, and vaccines, etc. Its usage has been in decline because of safety concerns. Since vaccine production is on the rise, its use may increase further in low-income and developing countries, as a cost-effective vaccine preservative. Further, Thiomersal is still being used as an essential component in various pharmaceutical preparations. In this light, the present study addresses its mechanism of toxicity in zebrafish and unveils a novel strategy for lessening its negative effects by conjugating cysteine to it, while retaining its antibacterial efficacy. We show that the mitochondrial membrane potential is destabilised by TM, leading to the induction of apoptosis. Interestingly, TM-cysteine conjugate (at a ratio of 1:1) showed no toxicity in zebrafish, whereas TM alone was highly toxic. Importantly, assaying for the bactericidal activity, tested using *Escherichia coli* (*E. coli*) and Methicillin-resistant *Staphylococcus aureus* (MRSA), revealed that the conjugate retains the antibacterial activity,

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demonstrating that the TM-cysteine conjugate is a safer alternative to TM as a vaccine preservative, and in all the other products that still use TM.

1. Introduction

Thiomersal is an organomercurial derivative of ethylmercury that has long been used in multi-dose vials of medicines and vaccines as a preservative due to its antibacterial and antifungal properties. Although the use of organomercurials such as TM in medical preparations is prohibited due to their toxicity in developed countries, these substances are still widely used in economically weaker nations. The main toxic ingredient in TM is mercury that is a highly toxic pollutant that has serious consequences for both humans and ecosystems (Bjørklund et al., 2017; Park and Zheng, 2012). Among various mercuric compounds the organomercury is the most dangerous form of the element because, being water-soluble, it can be readily absorbed through the skin (Park and Zheng, 2012) or along with food (Wagemann et al., 1998) and distributed throughout the body rapidly (Bjørklund et al., 2017; Yel et al., 2005). They conjugate with mostly thiol-rich compounds upon entering, then actively circulate all over the body, eventually reaching the brain (Tsakiris, 2018; Uchino et al., 1995). Therefore, organomercury can cause irreversible harm to various organ systems of the body, including the nervous system, the kidneys, and others (Yawei et al., 2021). Cases of mercury poisoning have been reported to be caused by the beauty cream, produced in Mexico, Crema de Belleza-Manning (containing mercury in the form of mercurous chloride or calomel, amounting to 6–10% mercury by weight) (Weldon et al., 2000). In most cases, however, mercury is not listed as an ingredient, and a recent study in India found that it is present in many well-known fairness creams (Gopinath et al., 2021; Kader Mohiuddin, 2019). Not only is this a modern problem in the cosmetics industry, but mercury has also been widely used, in untested ways, in traditional medicines in many parts of the world (Liu et al., 2008; Street et al., 2015; Yawei et al., 2021), such as, in the Latin Americas, from *botanicas*, under the name Azogue, through oral administration to treat constipation, colic, or stomach-ache (Kapp, 2014; Masur, 2011; Weldon et al., 2000). Many pharmaceuticals, such as antiseptics, antifungal agents, immunoglobulin preparations, antigens for skin tests, antivenins, nasal and ophthalmic products, dental amalgams, tattoo inks, vaccines, etc., still contain TM or other mercury preparations (Clarkson and Magos, 2006; Wu et al., 2008).

The recent COVID-19 pandemic has raised concerns that there may be an increase in the use of mercury-containing preservatives like TM during epidemic outbreaks. The WHO still recommends the use of vaccines containing TM for global immunization programs “because the benefits of using such products far outweigh any theoretical risk of toxicity” (WHO Technical Report Series, No. 926, 2004). Vaccines produced and sold in developed countries, like the United States of America, typically do not contain TM, but this is not the case in other parts of the world, such as Africa or Asia (Kollaritsch and Rendi-Wagner, 2013). One such example is the recombinant hepatitis vaccine named Heberbiovac HB which contains about 100 µg/ml TM in each vial (UNEP reports, 2020). Because of the poor logistics in these regions, TM is needed in rural areas, where transporting and storing vaccines is a major concern. However, there is much debate regarding the hazardous effects attributed to TM. While some studies found no link between TM and adverse health effects, others claim that it is completely safe for human use (Andrews et al., 2004; Price et al., 2010). However, according to a report by the United Nations Environment Programme (UNEP), as much as half of all vaccines manufactured each year are wasted or discarded due to poor storage conditions and inadequate infrastructure. For example, assuming a non-profit cost of around \$10 per vaccine, the current COVID-19 case, this spoilage costs more than a billion USD annually (UNEP reports, 2020). In this condition the use of TM could resolve

much of this problem.

In light of the conflicting reports, we sought to investigate the toxic mechanisms of TM in greater detail using zebrafish (*Danio rerio*) larvae as the model organism. Moreover, since it is still utilised as preservatives for some vaccines (Centers for Disease Control and Prevention [CDC], 2020) and other contemporary products, we sought to lessen its toxicity while preserving its antibacterial activity. In keeping with mercury species having a high propensity to combine with sulphur to form complexes (Velempini and Pillay, 2019; Wu et al., 2008), we show that TM forms a stable complex with cysteine and that this conjugate is far less toxic than TM alone, as observed from the enhanced larval survivability and lower DNA damage; without much compromising the bactericidal activity of TM, thus suggesting a superior alternative to TM alone.

2. Materials and methods

2.1. Reagents and software: Thiomersal (Cat No. 85090) was procured from Sisco Research Laboratories (purity 99%). The cyanin dye JC-1 (Cat. No. T4069), Rotenone (Cat. No. R8875), DCFDA (Cat. No. D6883), Ethidium Bromide (CAS. No. E7637), L-Glutathione (reduced) (Cat. No. G4251), L-Cysteine (Cat. No. C8755) were bought from Sigma-Aldrich. The other reagents used were Hydrogen Peroxide (Nice chemicals, Cat. No. H10683), SDS (Calbiochem, Cat. No. 428015), Polyacrylamide (MP biochemicals, Cat. No. 193982), Bovine Serum Albumin (Himedia, Cat. No. 9048-46-8), Collagenase (Gibco, Cat. No. 9001). γ -H2AX antibody (A300-081 A) was purchased from Bethyl Laboratories Inc. Human lung adenocarcinoma cell line, A549 was obtained from National Centre for Cell Science, Pune. BioRender was used for scientific illustrations.

Ethical approval

All experiments were done in accordance with the animal welfare guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Husbandry, 2021) [License No. CAF/ETHICS/963/2023].

2.1. Fish stock and embryo maintenance

Wild-type zebrafish were maintained in purified tap water at 28 °C with 14 h light and 10 h dark cycles, consistent with the zebrafish circadian activity. To collect eggs, the fish were allowed to spawn using the photo-induced breeding method (Bilotta et al., 1999; Dutta et al., 2022; Tsang et al., 2017). Fertilized embryos and larvae were maintained in E3 medium (5.03 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂·2H₂O, and 0.33 mM MgSO₄·7H₂O) for three days. All our experiments were performed at three days post-fertilization stages only (3 dpf).

2.2. Cell viability assay

The cell viability of human lung adenocarcinoma cell line (A549) cells in presence of TM and TM-cysteine conjugate were determined according to the article (Dutta et al., 2016) and the absorbance was noted at 595 nm using the M200 Tecan PRO, a high-performance multimode readers for a variety of biological applications like absorbance and fluorescence measurements, absorbance scans, excitation and emission scans etc.

2.3. Determination of viability of TM-exposed zebrafish larvae

To determine the toxicity, a minimum of 10 larvae of 3 dpf were incubated with different concentrations of TM at 28 °C for 24 h. For each concentration, three parallel experiments were done and the number of viable larvae were counted and studied. Heart rate was recorded using a stopwatch at 96 hpf under EVOS M5000 Microscope (Thermo Fisher).

2.4. Determination of intracellular ROS of zebrafish larva

The amount of intracellular reactive oxygen species (ROS) was measured using the dye 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) (Dutta et al., 2016). After washing with E3 medium the larvae were incubated with 10 µM DCF-DA in the dark for 30 min at 28 °C. The cells were then harvested using Trypsin (1X) and directly analysed by the flow cytometer (BD Accuri C6 Plus) using the FL1-A channel. The data were plotted as a histogram using BD Accuri C6 Software.

2.5. Determination of mitochondrial membrane potential of zebrafish larva

The mitochondrial membrane potential was measured by using a lipophilic cation, the 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1) probe (Smiley et al., 1991). A separate set of controls and treated larvae were stained with 1.5 µM of JC-1 reagent for 30 min, followed by rinsing the larvae with PBS and finally harvesting the cells, as mentioned above, in 1 ml PBS. The positive control cells were treated with 10 µM rotenone. The stained cells were immediately analysed by a flow cytometer using FL1-H vs FL2-H channel. The data were plotted as dot plots using BD Accuri C6 Software. Free swimming larvae were incubated with 1.5 µM of JC-1 for 30 min and then washed three times in embryo medium. Imaging was taken after 90 min of JC-1 incubation with the help of a Zeiss LSM 880 confocal microscope (Carl Zeiss AG).

2.6. Detection of cellular apoptosis

Apoptosis study was performed using the FITC-Annexin V detection kit according to the manufacturer's protocol (BD Pharmingen, Cat no 556547). After harvesting the cells, as mentioned earlier, cells were subjected to FACS analysis. Cells treated with 6 mM hydrogen peroxide (H₂O₂) were used as a positive control.

2.7. Comet assay

The comet or single-cell gel electrophoresis (SCGE) assay is a very sensitive test for the detection of DNA damage. The comet assay was done according to the method described previously (Jarvis and Knowles, 2003). Briefly, larval cells were isolated using 1X trypsin and mixed with molten 0.5% low-melting agarose. The mixture was immediately transferred onto a 1% agarose-coated slide followed by covering the slide with a coverslip. After the agarose solidified, the coverslip was gently peeled off and incubated with fresh lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base pH 10, 1% Triton X, and 10% DMSO) overnight at 40 °C. Following this stage, the slides were kept for electrophoresis at 100V for 10 min with freshly made electrophoresis buffer (pH > 13.0). Then the slides were dipped in the neutralizing buffer solution (400 mM Tris, pH 7.5) for 5 min. After this, the slides were further dipped in a solution containing Ethidium Bromide (0.5 µg/ml) for 10 min. The slides were washed thoroughly with distilled water and observed under a fluorescent microscope (EVOS-FL, Invitrogen) using a 10X objective.

2.8. Western blot

Total protein was isolated from 20 larvae, for each concentration of

TM tested, using a lysis buffer according to the manufacturer's protocol (Abcam 10X Cell Lysis Buffer: ab152163). The same amount of protein was loaded and separated on a 12% SDS-PAGE gel. The gel was then transferred into a polyvinylidene fluoride membrane and blocked with 5% BSA in TBST [Tris buffer, 20 mM; NaCl, 150 mM; Tween 20 detergent, 0.1% (w/v)] buffer for 1 h at room temperature. The membrane was then incubated with specific primary antibodies namely rabbit cleaved PARP (CST, Cat. No. 5625S), rabbit Caspase 3 (SCB, Cat. No. SC-7148), goat Tubulin (SCB, SC-12462) and rabbit Actin (Spring Bioscience, E3884) overnight at 4 °C, followed by incubation with HRP-conjugated anti-rabbit/anti-goat secondary antibodies (GeNei) for 1 h at room temperature. Images were taken using the Bio-Rad Gel Documentation system. Densitometric analysis was done by using ImageJ software to quantify the protein expression level. The blots are cropped from different parts of the same gel.

2.9. Mass spectroscopy

Thiomersal and L-Cysteine at a concentration of 1 mg/ml were prepared by dissolving the respective chemicals in ultrapure water (LC-MS grade, Sigma-Aldrich). Conjugate was prepared by taking 1:1 ratio of Thiomersal and L-Cysteine solution and were allowed to mix overnight on a magnetic stirrer. The resultant solution was subjected to lyophilisation to obtain a white powder. Mass spectra for all the samples were obtained on the Agilent LC (Agilent technologies 1260 Infinity) coupled to ESI Qtof mass spectrometer (Bruker Daltonics, Germany). The solutions were passed through a reverse phase C18 column at an infusion rate of 0.2 µL/min into the ESI chamber. The mobile phase, used for the same, consisted of water and acetonitrile with 0.1% formic acid. A linear gradient of acetonitrile from 5% to 95% was set in 50 min. Nebuliser pressure was kept at 26 psi, with a dry gas temperature of 220 °C and dry gas flow of 9 L. The full scan of the mass spectra was recorded in the *m/z* range [50–1000 Da] in positive mode. Moreover, the experiments were carried out more than once; when measurements were repeated, indistinguishable results were obtained. Only one set of reproducible data is reported here. ESI-MS spectra were acquired with tof software and analysed using software compass Data analysis 4.2 (Bruker Daltonics, Germany).

2.10. Whole-mount immunohistochemistry

Whole larvae, fixed with 4% paraformaldehyde, were rehydrated with PBS. The larvae were permeabilised with collagenase and blocked with 2.5% BSA for 2 h at room temperature, followed by which the larvae were incubated with γ-H2AX primary antibody (Bethyl laboratories, Catalogue No. A300-081 A) at a concentration of 1:5000 for overnight at 4 °C. After that, the larvae were stained with secondary antibody (anti-rabbit, Invitrogen) at a dilution of 1:100 and visualised with a Leica SP8 confocal microscope.

2.11. Swimming assay

To examine the swimming ability of zebrafish larvae, a swimming assay plate was developed using a plastic petri-dish of 90 mm × 15 mm in size. Concentric circles at 0.5 cm intervals of the radii were drawn at the bottom of 9 cm petri-dish and filled with 1X E3 medium. A swimming assay was carried out using 10 larvae and they were released one by one at the centre. Larvae move to the edge of the plate naturally or upon touching with a single hairbrush (maximum 3 times) to provoke an escape response from the centre. All the swimming experiments were recorded using a camera for further analysis. The total distance travelled by each larva, along with the time taken to achieve the task, was quantified.

2.12. Immunocytochemistry

A total of 50,000 cells were placed on a coverslip and permitted to attach overnight. Next day, IC₅₀ concentration of Thiomersal (200 ng/ml) and equivalent concentration of TM in conjugate (Thiomersal and L-Cysteine at 200 ng/ml) was applied on to the cells and incubated for overnight. The cells were washed with PBS, fixed in ice-cold methanol for 10 min and then blocked in 10% FBS in PBS in RT for 1 h. Cells were then incubated with rabbit primary Caveolin 1 antibody (Cloud-Clone Corp.) at 1:100 dilution for overnight at 4 °C. Following the washing step, the detection of antibody binding was carried out by incubating with anti-rabbit Alexa Fluor 488 at a concentration of 1:5000 for 1 h in darkness. Hoechst 33,258 (Invitrogen) dye at a concentration of 1:10,000 was applied to mark the nuclei of the cells for 20 min at RT. Images were obtained using a Zeiss Confocal 880-Multiphoton system. The fluorescence intensity was quantified using ImageJ software and results are expressed as mean fluorescence intensity (MFI).

2.13. Interactions between TM and its conjugate with BSA

To determine the interactions between TM and its conjugate with BSA various concentrations of TM and conjugate were mixed with a fixed amount of BSA (1 mg/ml) in 96 well plate and spectra was taken using the M200 Tecan PRO from 280 to 500 nm range. Both the cases milli-q water was taken as a reference control. Equivalent amount of TM was used in case of conjugate.

2.14. Protein-ligand docking

To determine the most likely position, orientation, and conformation in which a ligand will attach to a protein, we have used protein-ligand docking (Trott and Olson, 2010). Here we have docked two human proteins glutathione peroxidase 2 (GPX2) [PDB ID: 2HE3] and albumin [PDB ID: 1AO6] as receptors, separately to TM and cysteine-conjugated TM, as ligands. Water molecules were separated from the PDB files and polar hydrogens were added simultaneously to the same PDB file. On the other hand, PDB structures of ligand molecules were subsequently changed into. pdbqt format with the help of AutoDock Vina software. Now the final PDB of 1AO6 was enclosed in a grid box and the dimensions of the grid box were fixed at 114 × 108 × 126 with a grid spacing value of 0.375 Å. The centre of the grid was positioned at −12.508, −0.032, and −7.904 Å respectively. The final PDB of 2HE3 was enclosed in a grid box and the dimensions of the grid box were fixed at 98 × 86 × 92 with a grid spacing value of 0.375 Å. The centre of the grid was positioned at −11.152, −1.072, and −6.031 Å respectively. The preferred binding mode between the receptor and the selected ligand molecule was determined based on the results of AutoDock Vina's docked conformations, considering the scores with the lowest energy. The lowest energy docked conformations, obtained from the AutoDock Vina scoring function were considered as the preferred binding mode between the two receptors and the respective selected ligand molecules. The Lamarckian genetic algorithms (GA) modules available with AutoDock were employed to execute present ligand docking calculations. The AutoDock programme default settings were chosen to indicate additional parameters (Morris et al., 1998; Sanner, 1999; Trott and Olson, 2010). The Python Molecular Viewer application was used to view the results of the calculation (Stefano Forli, Olson Molecular Graphics Laboratory, The Scripps Research Institute, San Diego, CA).

2.15. Statistical analysis

All experiments were done independently three times with different batches of larvae. Data points were obtained from the mean of at least three replicates. Statistical significance, if any, between the experimental group and control group data, was determined by analyses of variance and the results are presented as the mean ± standard error of

the mean (SEM). The significance was defined as $p < 0.05$ (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = non-significant).

3. Results

3.1. Thiomersal exhibits toxicity towards developing zebrafish larvae and A549 cells

TM is present in vaccines administered to children (World Health Organization, 2011; Serum Institute of India, 2023), although the effect of TM on developing animals is not well understood. So, we evaluated this by exposing zebrafish larvae to various concentrations of TM for 24 h. But before that, we assessed the toxicity of TM on the lung carcinoma cell line (A549). The study revealed that TM exhibited high toxicity, with LC₅₀ and LC₁₀₀ values of 200 ng/ml and 700 ng/ml, respectively (Supplementary Fig. S1). In our zebrafish larval experiment (Fig. 1A), no mortality was noted up to 250 ng/ml of TM, but beyond this concentration, larval mortality was observed. After 24 h exposure, the determined LC₅₀ and LC₁₀₀ values were 750 ng/ml and 2000 ng/ml respectively. At these concentrations of TM, only 53.33% ± 3.33 and 3.33% ± 3.33 of the larvae were found to be alive. Interestingly, all the dead larvae showed no deformity or deterioration of body parts, except a visible blood accumulation in the heart (Fig. 1C, inset), with a direct correlation between the volume of blood with the increasing TM concentration in the medium (Fig. 1C, inset). In addition, it was also found that as TM concentration increased, the heart rate of the larvae decreased noticeably. From a baseline of 145 ± 1.86 beats per minute (bpm), the heart rate dropped to 78.4 ± 12.14, 12.57 ± 8.16, and 0 bpm at the concentration of 750, 1000, and 2000 ng/ml of TM, respectively (Fig. 1B). The observed accumulated blood in the heart could be an indication that the larval heart rate has slowed to the point where it is unable to pump blood (Fig. 1C). Our results corroborate previous research showing that exposure to mercury causes alterations in cardiac rhythmicity and function, and is linked to cardiovascular illnesses (Dórea, 2017; Wu et al., 2008). Elevated concentrations of TM (exceeding 700 ng/ml) and brief exposure periods (24 h) result in the debilitation of larvae. However, those larvae appearing viable within the initial 24 h may not endure beyond that timeframe. Given our study's focus on the 24-h period, we classified larvae displaying any signs of vitality as "live." Consequently, 25% of larvae demonstrated survival with heart rates around 12 bpm at 1000 ng/ml of TM. Following the same reasoning, we observed that only a minor fraction of larvae, approximately 1–2%, can endure in extremely high TM concentrations. Achieving LC₁₀₀ or LC₉₈ values demands significantly higher TM amounts, specifically 2000 ng/ml.

3.2. Thiomersal induces apoptosis by destroying mitochondrial membrane potential

Organomercury exposure induces oxidative stress, which has been linked to apoptosis (Widiastuti et al., 2020; Zulaikhah et al., 2020). Therefore, to validate this phenomenon in our model system, the levels of reactive oxygen species (ROS) in larval cells exposed to TM was measured at three different concentrations (viz., 500, 700, and 800 ng/ml), encompassing both the lower and higher values of LC₅₀. Since only a slight change in intracellular ROS was observed after 24 h of TM incubation (Fig. 2A), we decided to investigate the kinetics of the ROS induction at different time points following TM administration (at 750 ng/ml TM). We found that there was a steady increase in ROS concentration with time, with a maximum at 6 h, followed by a decay in ROS levels until 24 h post TM exposure (Fig. 2A).

TM has been reported to damage the cell membrane and its permeability, and, consequently, the mitochondrial membrane potential (MMP) (Baskin et al., 2003). To validate these findings *in vivo*, and correlate them to results from the previous experiment (Baskin et al., 2003), we used flow cytometry and confocal microscopy with JC-1 dye

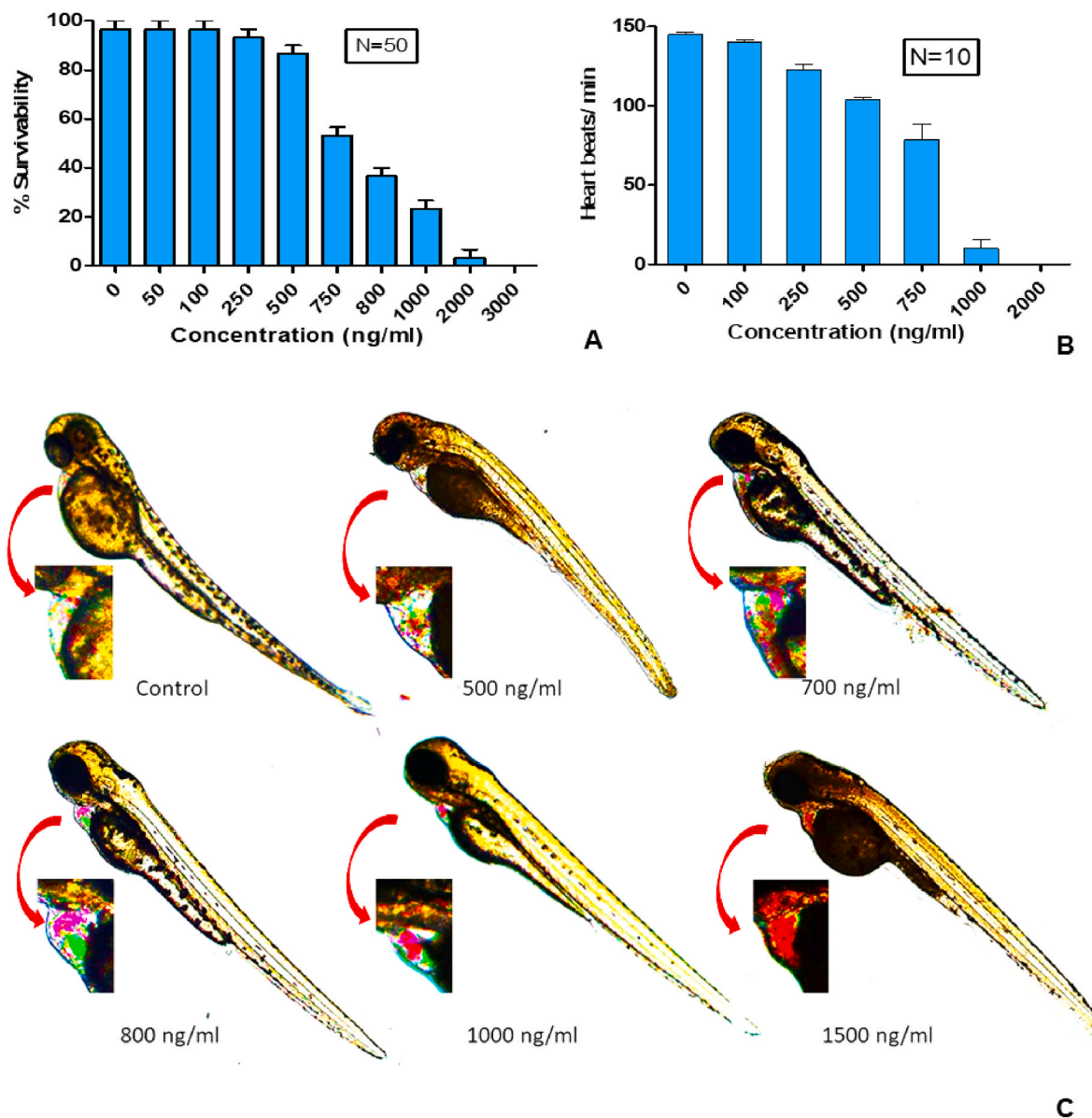


Fig. 1. Thiomerals toxicity on developing zebrafish larvae. A: Survivability of larvae in various concentrations of TM. B: Comparative cardiac rate (in bpm) of zebrafish larvae in various concentrations of TM. C: Image of larvae in various concentrations of TM, with inset showing the accumulated blood in the larval heart. N represents the number of larvae used in each experimental repetition.

to examine changes in MMP upon TM exposure. The percentage of cells containing depolarised mitochondria rose from 55.8% in control larvae to 70%, 71.9%, and 78.4% for 500 ng/ml, 700 ng/ml, and 800 ng/ml of TM exposure, respectively (Fig. 2B). We used rotenone as a positive control. Rotenone, commonly used as an insecticide and serves as an inhibitor of mitochondrial complex I. This property leads to mitochondrial dysfunction, making it a suitable choice as the positive control in cellular experiments assessing mitochondrial membrane potential (Colpo et al., 2017; Heinz et al., 2017).

The presence of a substantial percentage (55.8%) of depolarization in the mitochondrial membrane potential (MMP) of control larval cells raises questions. To investigate MMP changes, it becomes essential to extract cells from zebrafish. Despite the use of tissue-disintegrating enzymes to facilitate this process, the extraction proved to be less than seamless, requiring additional mechanical force for cell isolation. Unfortunately, this introduced unavoidable stress to the cells, adversely affecting their overall health. Particularly sensitive to such changes, mitochondria reflected alterations induced by these stressors.

Consequently, the isolated cells did not maintain their optimal health, resulting in MMP depolarization in the control larvae. The heightened depolarization in the control larvae can be attributed to the stresses incurred during the isolation process. However, even with this increased depolarization in the control group, the test MMPs exhibited an even more significant depolarization. This indicates that the experimental conditions, notably the application of Thiomerals (TM), have a detrimental impact on mitochondria, surpassing the stress-induced depolarization observed in the control group.

The findings from our confocal microscopy observations supported the FACS data as well. Green fluorescence, indicating depolarised mitochondria, increased over time as TM concentration rose, a sign of mitochondrial dysfunction (Fig. 2C), resulting in a complete loss of cellular energy.

TM has been reported that it induces apoptosis via the induction of DNA damage, effector Caspase activation, and membrane damage (Baskin et al., 2003). FACS data with Annexin V and Propidium Iodide demonstrated a gradual increase of total dying cells in the TM-treated

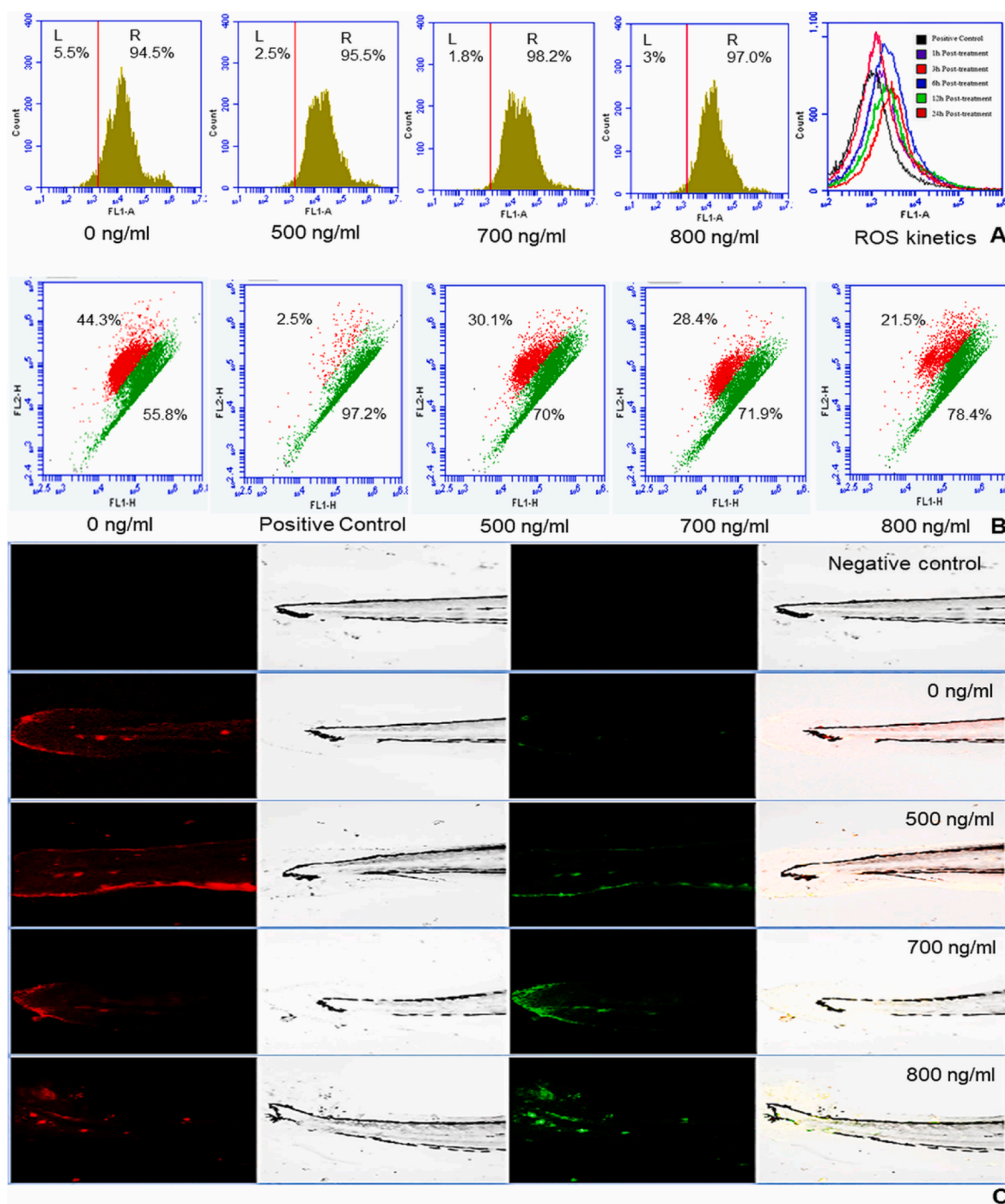


Fig. 2. Depolarization of MMP in TM-exposed Zebrafish larva. A: Flow cytometry result on ROS level in larval cells. B: Flow cytometric detection of depolarization of MMP in larval cells. C: Confocal images of the intact larva having depolarised MMP.

populations (Fig. 3A and B), corroborating the previous report (Baskin et al., 2003). In Fig. 3B, the percentage of total number of dying cells was determined by summing the percentages of early apoptotic, late apoptotic, and necrotic cells. This involved aggregating the percentages of cells found in quadrant Q1-LR, Q1-UR, and Q1-UL of Fig. 3A whereas Q1-LL represent the healthy population of cells. The total dying cells was found to be around 38.8%, 50%, and 57.3% at 500 ng/ml, 700 ng/ml, and 800 ng/ml of TM concentration, respectively (Fig. 3B). The comet assay results further consolidated the FACS data on apoptosis (Fig. 3C and D). The comet's tail length (CTL) for control cells was 8.33 μ m, increasing to 27.65 μ m, 57.2 μ m, and 156.7 μ m when exposed to TM at 500, 700, and 800 ng/ml, respectively (Fig. 3C and D). These findings

confirmed that DNA degradation, a hallmark of apoptosis, occurs as a result of TM treatment. Apoptosis was further confirmed by Western blot using antibodies against cleaved poly (ADP-Ribose) polymerase (PARP) and Caspase-3. Our study shows that concentrations of both cleaved PARP and Caspase-3 increased by many folds after 24 h of TM treatment. Fold changes for cleaved PARP were approximately 0.87, 1.44, and 1.07 at 500, 700, and 800 ng/ml TM, and for Caspase-3 these values were approximately 0.305, 0.598, and 1.055, respectively (Fig. 3E and F).

3.3. Cysteine and TM combine to form a stable compound

Mercury, being a thiophilic compound, has a high affinity for sulphur

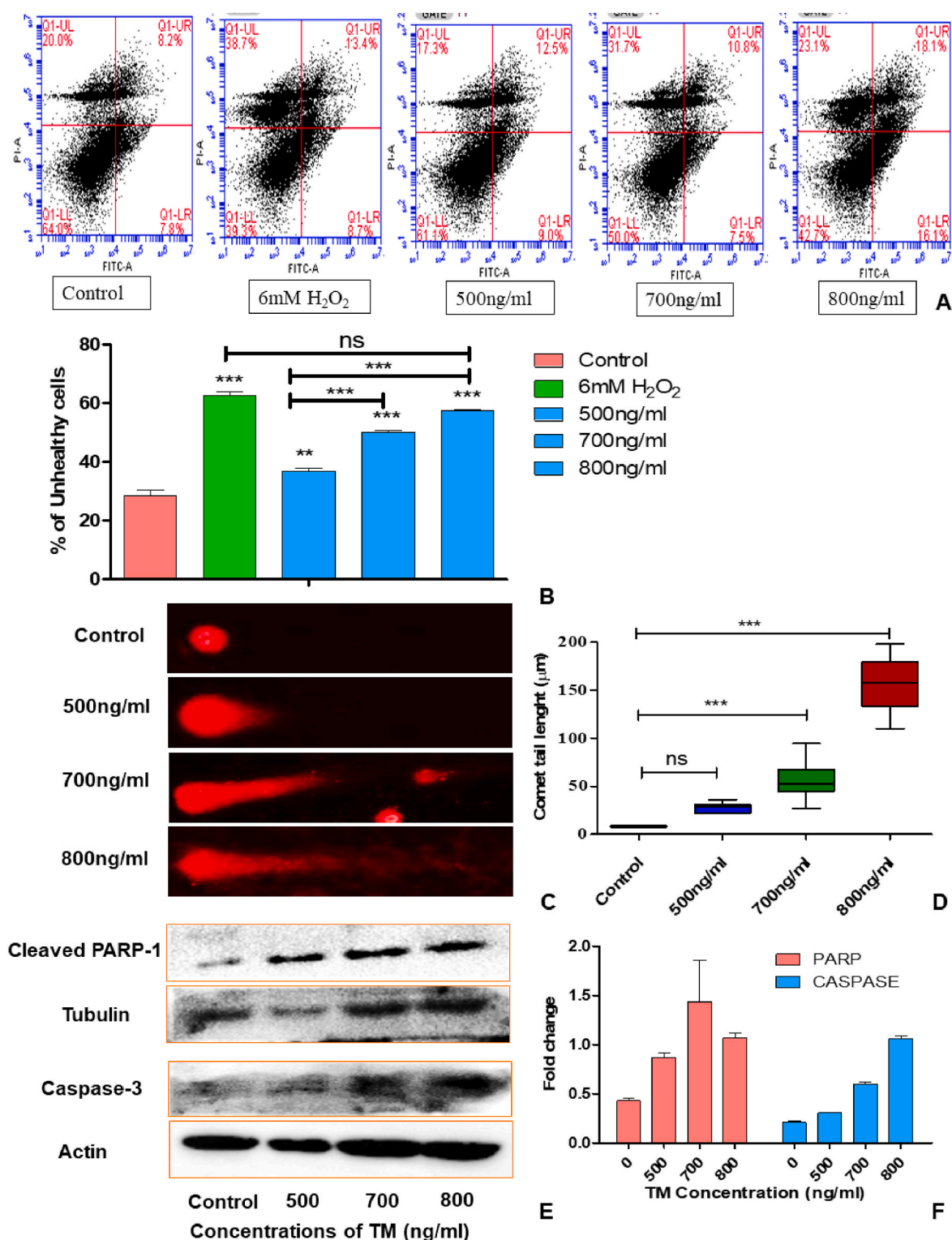


Fig. 3. TM-mediated cellular apoptosis in zebra fish larvae. **A:** Flow cytometric detection of apoptosis. **B:** Graphical quantification of the percentage of dying cells (which includes early apoptotic, late apoptotic, and necrotic cells) in the larvae. **C:** Comet assay to detect the level of DNA degradation. **D:** Mean comet's tail length (in μm) in larval cells. **E:** Western blot analysis of cleaved PARP and Caspase-3. **F:** Densitometric analysis of the Western blot for quantitative detection of cleaved PARP and Caspase-3. (** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant).

in its various forms, and, for example, forms 'mercaptan' ('seizing mercury' in Latin) (Melnick et al., 2010). Between the two sulphur-containing amino acids, we chose cysteine as a conjugator of TM; because, unlike methionine, cysteine is rapidly ionised with a negatively charged thiolate group, which boosts its reactivity with TM

(Poole, 2015). Cysteine, by virtue of being an amino acid, is not expected to increase the toxicity of TM. Using Electrospray ionisation mass spectrometry (ESI-MS), we have shown that TM forms a stable complex with cysteine (Fig. 4C). Along with the probable expected isotope distribution pattern, peaks at m/z 122.02 Da for L-cysteine (Figs. 4A) and

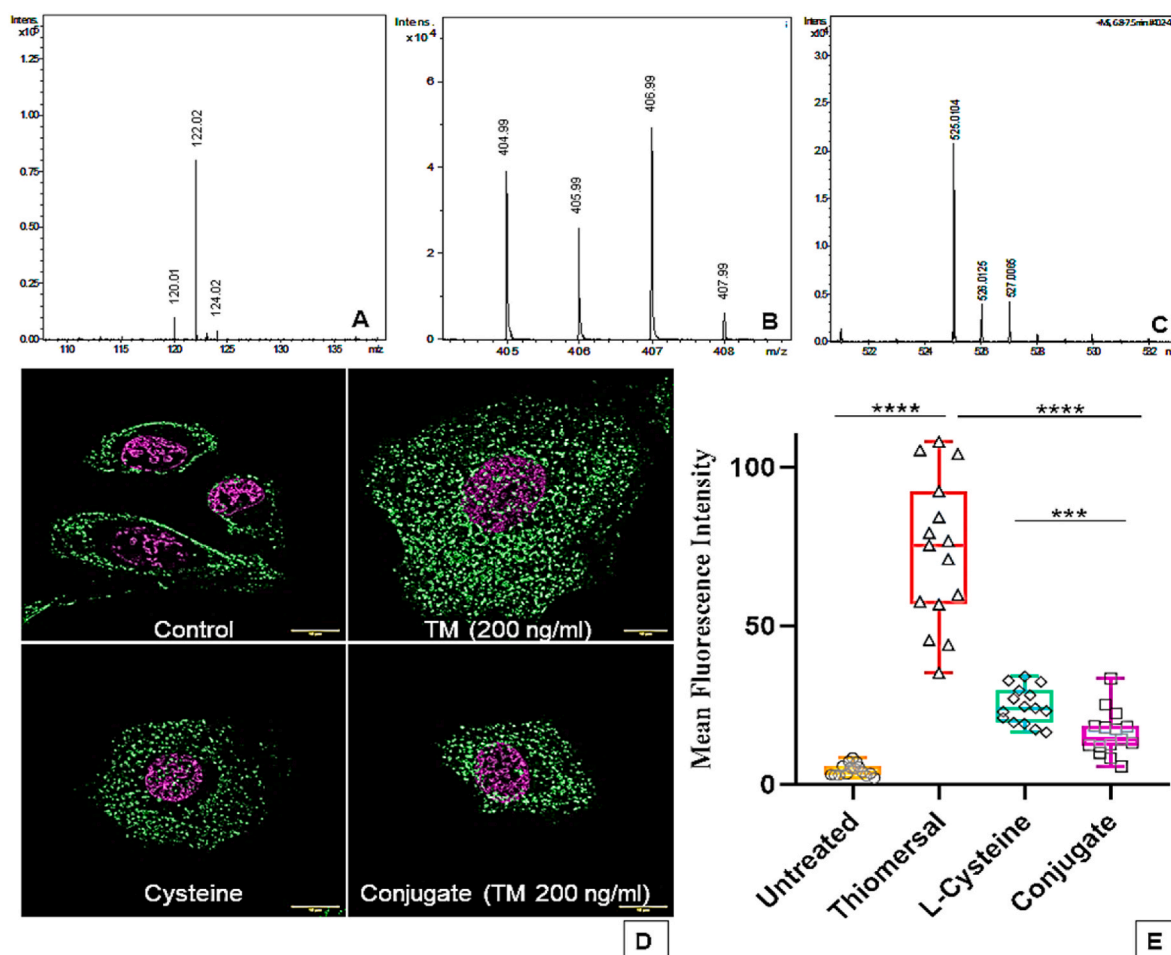


Fig. 4. ESI-MS spectra of Cysteine, TM and Conjugate and Caveolin 1 expression in A549 cells. Spectral analysis shows that TM and Cysteine form a stable conjugate. A. l-cysteine. B. TM. and C. Conjugate. D. Immunocytochemistry of A549 Cells for Caveolin 1, indicated as green (alexa-fluor 488). E. Expression pattern of Caveolin 1. The TM treated group showed increased expression of Caveolin 1, but cysteine and conjugate group shows lower Caveolin 1 levels like that of untreated control. (** = $p < 0.001$, **** = $p < 0.0001$, ns = non-significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

405.99 Da for TM (Fig. 4B), and a peak at m/z 525.01 Da for the conjugate (Fig. 4C) was seen.

3.4. Endocytosis of TM is facilitated by caveolin 1

To investigate the intracellular vesicular trafficking of mercury compounds such as TM, we focused on the expression of Caveolin 1. Our study revealed intriguing insights: upon treatment of A549 cells with TM (IC_{50}), there was a noticeable upregulation in Caveolin 1 expression as compared to both the control group and the conjugated group (Fig. 4D and E). Interestingly, this suggests that when TM is conjugated with cysteine, its cellular uptake is potentially hindered, as evidenced by the decreased expression of Caveolin 1.

3.5. Cysteine conjugation to TM that attenuates TM-mediated toxicity

In order to evaluate the neutralizing effectiveness of cysteine when conjugated with TM, we focused on the highest observed toxic concentration of TM, referred to as LC_{100} (2000 ng/ml). This concentration was chosen as the focal point for assessing how well cysteine could mitigate the adverse effects of TM toxicity in zebrafish. When TM was combined with varying doses of cysteine, we found a significant increase in larval survival (Fig. 5A). The larval survival increased from 2% to 66% when cysteine was added to the medium in an equal concentration to that of the TM, i.e., 2000 ng/ml (TM:cysteine = 1:1). Beyond that

concentration, survivability began to decline. However, with all cysteine co-treatments, larval survivability remained better than that with TM treatment alone. Now questions may arise regarding the decline in larval survivability above a cysteine concentration of 2000 ng/ml. It is widely acknowledged that among various amino acids, cysteine is notably detrimental to cells, particularly impacting mitochondria. It impedes mitochondrial respiration by limiting intracellular iron availability through an oxidant-driven mechanism. The observed higher larval mortality beyond a cysteine concentration of 2000 ng/ml may be attributed to elevated cysteine concentrations, which could introduce toxicity alongside TM. To substantiate this hypothesis, we conducted toxicity tests in environments with high cysteine concentrations, and our findings indeed indicate that elevated cysteine concentrations induce toxic effects to larvae (Hughes et al., 2020; Osman et al., 1997) (Fig. S2). Interestingly our cell culture study also reflects analogous outcomes. The MTT data shows TM alone exhibits an LC_{50} value of approximately 200 ng/ml, whereas the conjugate demonstrates a notably higher LC_{50} value of about 600 ng/ml of TM. (Fig. S1). Surprisingly, adding cysteine not only increased the larval survivability but also boosts their health in general. To learn more about this phenomenon, we analysed the escape behaviour of larvae treated with TM, and TM-cysteine conjugate. Indicative of healthy physiology, the larval escape is an involuntary response to any threatening or stressful situation (Basnet et al., 2019; Kalueff et al., 2013). Thus, larvae that perform better in such tests are considered most likely to be healthier. The conjugate-treated group

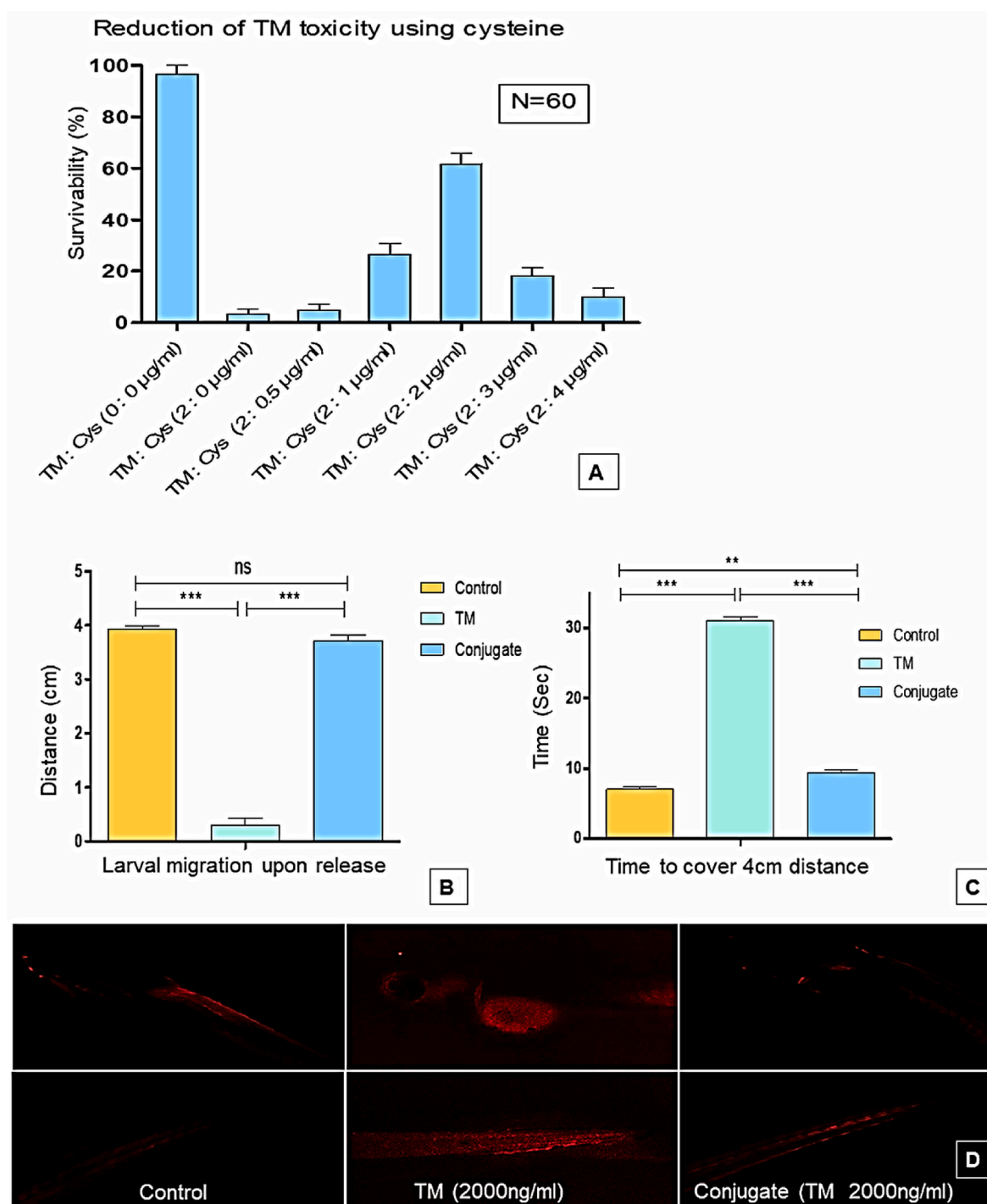


Fig. 5. Cysteine can mitigate TM-mediated toxicity. A: larval survivability in TM-cysteine conjugate at concentrations equivalent to TM's lethal dose. B: A comparative graph showing the distance covered by larva upon release (maximum of 4 cm). C: A comparative graph showing the time taken by the larva to cover 4 cm distance. D: Immunohistochemical staining of γ -H2AX, the two figures represent the head (the upper row) and tail region (the lower row) of larva, to represent the level of DNA degradation in them. (** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant).

exhibited a significantly more robust escape response and travelled a greater distance in a shorter amount of time (Fig. 5B and C), almost identical to that of the untreated control group. Contrarily, TM-treated larvae were in poor physiological condition, as evidenced by a nearly non-existent escape response (supplementary video S4). Thus, conjugating TM with cysteine in equal amounts effectively nullifies its toxic effects. We then investigated whether the addition of cysteine in the medium could also stop TM-mediated DNA degradation in the larva. For this purpose, the level of γ -H2AX in the larva was checked, since γ -H2AX

is a very specific and sensitive marker for DNA damage (Riffle et al., 2017). The TM-treated groups had more DNA damage than both the control and the cysteine-conjugated TM groups (Fig. 5D).

Next, to decipher the molecular mechanism underlying the toxicity of TM, we investigated the interaction between TM and its conjugate with BSA. The interaction was assessed by monitoring BSA absorption against gradual increment of TM concentration. The results revealed a bathochromic shift, or red shift, in the spectrum, indicating a shift of the UV absorption peak towards a higher wavelength with each successive

increment of TM concentration. This shift may be attributed to a strong interaction between TM and BSA. Fig. 6A illustrates these robust interactions between the TM and BSA. Conversely, when BSA interacts with the conjugate, the pattern of UV absorption spectrum got completely lost (Fig. 6B). This suggests that, due to cysteine conjugation,

TM was no longer able to interact freely with proteins, specifically with BSA. Furthermore, we used molecular docking with two important human proteins, albumin (PDB ID: 1AO6) (Fig. 6C and D) and glutathione peroxidase 2 (GPX2) (PDB ID: 2HE3), (Fig. 6E and F) to test the hypothesis that conjugation of cysteine to TM lowers its affinity for

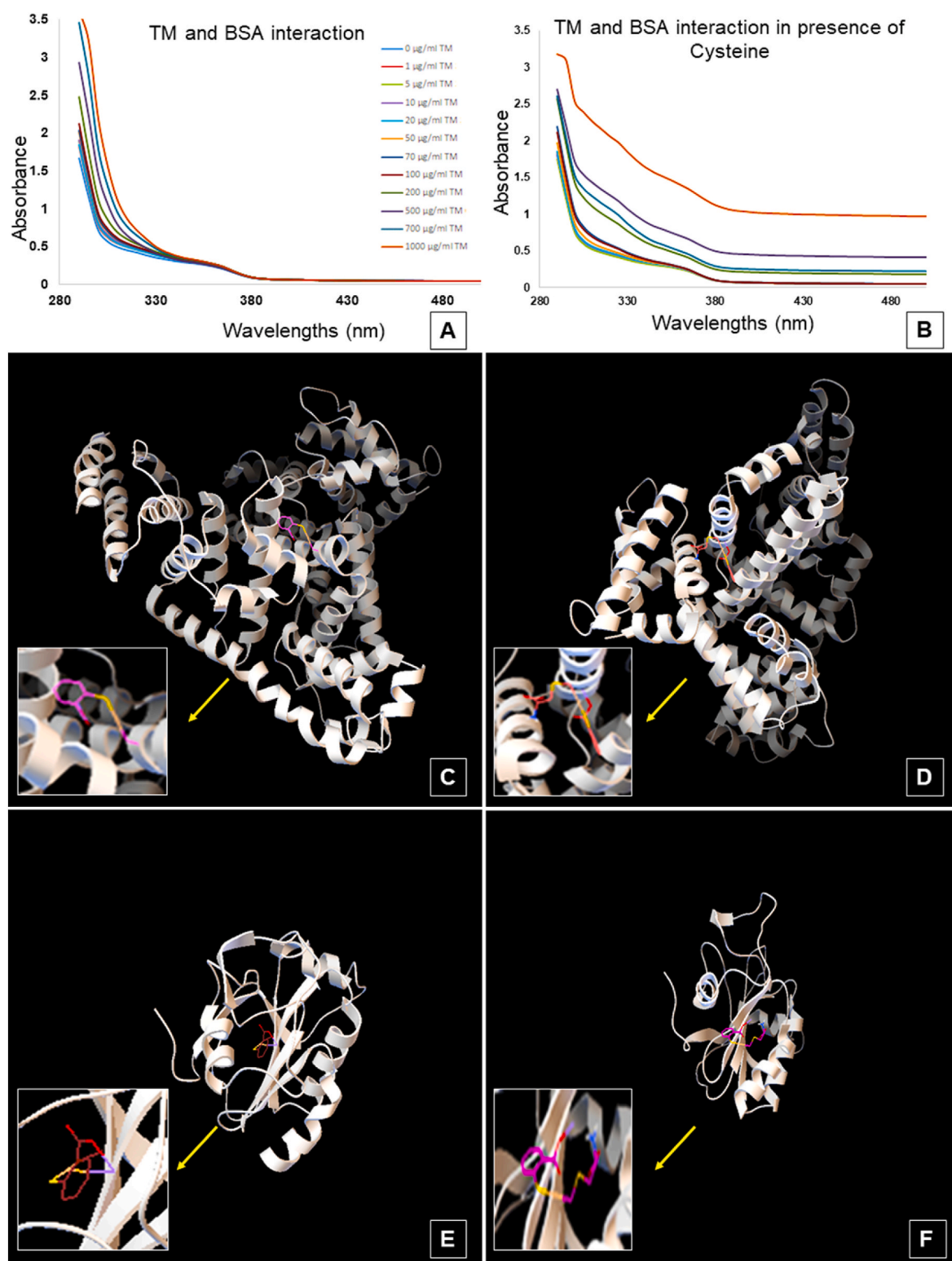


Fig. 6. Interaction of TM with proteins. A: Interaction of TM with BSA is spontaneous, revealing that the TM may interact with any protein inside the cell. B: Interaction of conjugate with BSA, showing that TM-cysteine conjugate hinders the spontaneous interaction of TM with BSA. C–F: *In silico* predicted interaction C: of TM with human albumin, D: of TM-cysteine conjugate with human albumin, E: of TM with human GPX2, F: of TM-cysteine conjugate with human GPX2. inset at C, D, E, and F showing the interaction pattern in enlarge form.

proteins. We found that the Gibbs free energy (ΔG) for the interaction of TM with albumin and TM with GPX2 was -7.8 kcal/mol and -6.9 kcal/mol, respectively, whereas, upon cysteine conjugation, the affinity for protein binding was found to be much lower. The ΔG for cysteine-conjugated TM with albumin and with GPX2 was found to be -3.5 kcal/mol and -3.3 kcal/mol, respectively. The possible ΔG and RMSD values of the interaction are provided in the [Supplementary Table 1](#). The amino acid residues involved in the interactions are displayed in [Supplementary Fig. S3](#). We found that when TM interacts with proteins on its own, it interacts strongly with a broad range of amino acids, but when the conjugate interacts, the interaction patterns change appreciably, with most of the interactions becoming unfavourable.

As cysteine conjugation mitigates the toxic effects of TM, the bactericidal potential of the resulting conjugate was investigated against Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* (*E. coli*). The outcomes regarding the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are presented in [Supplementary Table 2](#). For TM the MIC of *E. coli* and MRSA was found to be 0.5 $\mu\text{g/ml}$ and 1.5 $\mu\text{g/ml}$ and MBC was found 1 $\mu\text{g/ml}$ and 3.5 $\mu\text{g/ml}$. For conjugate the observed MIC of *E. coli* and MRSA was 0.5 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$ and MBC was 2 $\mu\text{g/ml}$ and 6.5 $\mu\text{g/ml}$. These results indicate that the conjugate exhibits a robust bactericidal activity which is comparable to that of the original compound. This implies that the addition of cysteine through conjugation not only mitigates the toxic effects of TM but also preserves its bactericidal efficacy.

4. Discussion

Mercury is a well-known toxic material that is known to affect almost all human organ systems. Two of the most devastating global incidents of mercury toxicity were the Minamata Bay tragedy in Japan in the 1950s ([Clifton, 2007](#)) and the one in Iraq in the 1970s ([Greenwood, 1985](#)). Among the various forms of mercury, organomercury is the most toxic because of its higher water solubility, and, therefore, can easily enter the bloodstream. TM is one such organomercury that is still used in many modern products. The clinical effects of lower doses of mercury remain controversial ([Vaccines, 2016](#)), prompting us to investigate the detrimental effects of TM on the biological system. According to the cell culture ([Fig. S1](#)) and zebrafish larval experiment ([Fig. 1A](#)) we discovered that TM is extremely toxic. [Supplementary Fig. 1](#)

We found that toxicity induced by TM follows a pattern in which initially, it increases intracellular reactive oxygen species (ROS), followed by mitochondrial damage, and finally, cell death occurs through apoptosis. Interestingly, ROS escalation was not constant. We observed a steady increase in ROS concentration with time up to 6 h, followed by a decay in ROS level ([Fig. 2A](#)). Similar kind of observations were also found in many articles ([Leveque et al., 2023](#); [Sharafutdinov et al., 2019](#)) but without a comprehensive explanation. So, we hypothesize that the observed decay in ROS levels could be attributed to multiple factors. In particular, mitochondria, which generate nearly 90% of cellular ROS, play a crucial role in metabolizing and oxidizing drugs or compounds ([Murphy, 2009](#); [Tirichen et al., 2021](#)). Consequently, ROS emerges as a byproduct of these mitochondrial processes. It is noteworthy that if mitochondria undergo damage during these metabolic activities, the presence of ROS within the cell may diminish. Paradoxically, even in instances of severe cellular damage, the decline in observable ROS could be indicative of compromised mitochondrial function. The gradual loss of mitochondrial function, as indicated by compromised mitochondria after TM treatment ([Fig. 2B](#)), may contribute to the diminishing ROS levels over time. Furthermore, it is plausible that the cellular response to the ROS peak involves activation of compensatory mechanisms, facilitating a return to homeostasis. Further investigation is warranted to elucidate the intricacies of these dynamics and the interplay of various cellular processes in governing the observed ROS fluctuations.

The FACS and confocal imaging for assessing the mitochondrial membrane potential (MMP) indicate that TM destabilises the MMP

([Fig. 2B & C](#)). These results would explain the significant decrease in heartbeat rate and extreme weakness in the TM-exposed fishes. Therefore, it can be inferred that the depolarization of the mitochondrial membrane and reduced ATP production, set off a 'domino' effect that impair the body movements, causing an extreme weakness in fish, and exhausting the heart muscle fibres, ultimately culminating in cardiac tamponade ([Fig. 1B & C](#)).

With the defective mitochondria, cells find it difficult to meet their energy needs, resulting in their progression toward death through the apoptotic cascade ([Sharpe et al., 2012](#); [Woo et al., 2006](#)) ([Fig. 3A & B](#)). With mitochondrial damage there is an activation of Caspase-9 ([Woo et al., 2006](#)), which can cleave the effector Caspase-3, inducing apoptosis. Cleaved Caspase-3, in turn, cleaves downstream target proteins, including PARP, a readout for apoptosis ([D'Amours et al., 2001](#); [Sharpe et al., 2012](#)) ([Fig. 3E & F](#)). The upregulation of Caspase-3 and cleaved PARP at higher TM concentration corroborates with the higher irreversible DNA damage, as seen with greater comet tail length ([Woo et al., 2006](#)), another hallmark of apoptosis ([Fig. 3C & D](#)).

Next, we showed that TM can strongly interact with model proteins like BSA, human albumin and GPX2 ([Fig. 6A, C and E](#)). This finding suggests that it is likely that TM can bind and lead to the destruction of multiple vital cellular proteins and enzymes in the cell. In addition, TM is known to cause toxicity by interacting with all cellular thiols ([Yel et al., 2005](#)). On the other hand, approximately half of the world's vaccine supply is wasted because of inadequate storage and infrastructure, according to a 2020 report by UNEP. However, before utilizing TM as a preservative, it is essential to discover methods to counteract its toxic effects. So, to nullify TM's toxicity we capitalised the thiophilic activity of TM by co-opting the thiol side chain in cysteine. In fact, mercury, part of several preparations in Ayurvedic medicine in India, is typically rendered harmless (*samskara*), by converting it to a medicinal sulphide form ([Yadav and Chaudhary, 2018](#)). Cysteine was chosen, between the two common sulphur-containing amino acids, due to its high reactivity to TM, in comparison to methionine, and, by virtue of being an amino acid, its low risk of triggering cytotoxicity ([Poole, 2015](#)). Pre-mixing of TM with cysteine handicaps the ability of TM to interact with BSA or human albumin or human GPX2 ([Fig. 6B, D & F](#)). As cysteine strongly binds with TM, forms a stable TM-cysteine conjugate ([Fig. 4C](#)), it could reduce the amount of TM, free in the medium to interact with other cellular components. So, the reacting groups of TM become sequestered in conjugated form, and TM becomes less toxic. This diminished toxic effect can also be seen in [Fig. 5A](#) as well as in the γ -H2AX assay ([Fig. 5D](#)), where we show that cysteine addition hinders the DNA damage created by TM and improve overall larval physiology ([supplementary video S4](#)). Upon conjugation with cysteine, the toxicity of TM is further effectively diminished. The cysteine-conjugated TM exhibits diminished endocytosis when compared to its free form. The free TM triggers an increased expression of Caveolin 1, resulting in an elevated intracellular influx of TM, as evidenced by the data presented in [Fig. 4D and E](#). On the contrary, the conjugate displays a reduced level of Caveolin 1 expression and, consequently, a decreased intracellular uptake of TM. Although neutralizing the toxicity does not completely diminish the bactericidal activity of TM. The conjugate retains an effective bactericidal efficiency and its comparable to TM ([Supplementary Table 2](#)). [Fig. 7](#) provides a comprehensive illustration of the intricate mechanism wherein the TM exhibits facile cellular entry via Caveolin 1-mediated endocytosis. Upon internalization, TM dynamically engages with many cellular components and proteins leading to DNA damage and apoptosis while conjugate penetrates the cell very weakly and even if they enter the cell, they are less likely interact with proteins and other cellular components making conjugate a safer alternative to TM.

This discovery aligns with our objective to unearth a less toxic TM variant while barely hampering its intrinsic antimicrobial properties. Thus, in this way one can reframe TM to reduces its toxicity without significantly compromising its inherent antimicrobial qualities,

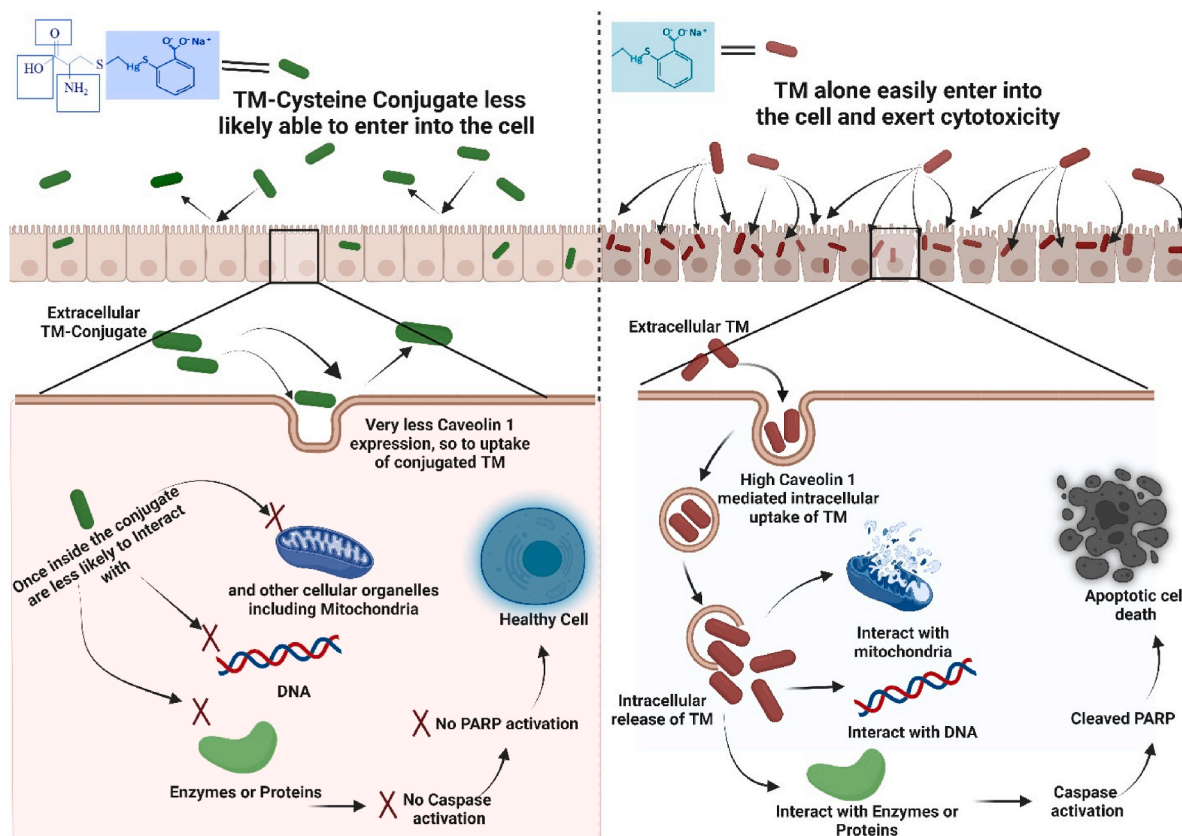


Fig. 7. Possible intracellular role played by TM and TM-cysteine conjugate.

resulting in an undoubtedly more efficacious solution.

TM interacts strongly with intracellular proteins and enzymes, interfering with their function, which becomes catastrophic for the cell. The overall effect includes damages to mitochondria resulting in cell death via apoptosis. On the other hand, conjugates containing cysteine protect cells from TM-mediated toxicity because of the strong interaction between cysteine and TM, which results in a stable compound that is no longer able to interact with intracellular proteins or enzymes.

CRediT authorship contribution statement

Debanjan Dutta: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sautan Show:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Anabadya Pal:** Writing – review & editing, Software, Data curation. **Anifowoshe Abass Toba:** Methodology. **Mahadesh Prasad Aj:** Methodology. **Upendra Nongthomba:** Writing – review & editing, Supervision, Project administration.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to improve language. After using this service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2023.141070>.

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