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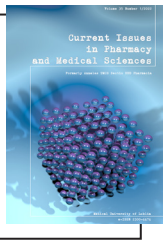

L-cysteine sequestering methyl glyoxal prevents protein glycation: a combined *in vitro* and *in silico* evaluation

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ABSTRACT

Reactive carbonyl species such as methylglyoxal (MGO) act as potent glycating agents that are implicated in several oxidative stress-induced diseases, including, but not limited to, cancer, diabetes and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. In the present study, a combined approach of initial computational studies and *in vitro* analysis was utilized to investigate the therapeutic benefit of L-cysteine (CYS), a thiol-containing compound. Based on the reactive analysis using global and local density functional theory (DFT) descriptors, the data reported here has revealed that CYS matches its electron-donating capacity with the electron acceptor, methylglyoxal, making the former a good candidate sequestering agent for the latter. Furthermore, *in vitro* analysis shows that CYS exhibits significant inhibitory effects towards damaging protein structural alteration and formation of advanced glycation end products (AGEs). Such assessment came about through Congo red binding Assay and Antiglycation Assay using BSA-MGO and BSA-glucose model systems. Additionally, CYS also demonstrates a significant protective effect towards MGO-induced oxidative stress in mouse C2C12 myotubes. Therefore, this thiol-containing compound is a promising therapeutic tool for several glycation or AGEs-induced pathological conditions. The use of more relevant cellular and animal models of desired disease studies is recommended to better understand the mechanistic basis of CYS's potential therapeutic effect. This study utilizing the combined approach of computational and in-vitro analysis, provides helpful data to maximize the potential impact of CYS by designing related molecules that could serve as novel treatment strategies that are effective, safe, and accessible to all in the future.

INTRODUCTION

Reactive carbonyl species (RCS) are a class of byproducts produced from exogenous and endogenous oxidation [1]. RCS owe their high reactivity to nucleophilic sites found in proteins, nucleic acids, amino phospholipids and other macromolecules to their electrophilic carbonyl moiety. RCS can exert their detrimental cellular effects by increasing ROS production, thereby forming a vicious cycle of ROS and RCS production [2]. These results in cytotoxicity and the ability to form different types of adducts and crosslinks that are connected with several chronic diseases such as cancer [3], diabetes [4], pulmonary and neurodegenerative diseases (e.g., Alzheimer's disease and Parkinson's disease) [5]. As key reactive intermediates of carbonyl stress, they induce damage to proteins and enzymes via glycation, forming AGEs [6]. Although AGEs are produced endogenously in the body, exogenous sources such as smoking and consumption of browned food contribute to the body pool. Therefore, when the AGEs pool in the body rises above physiological levels, different pathological conditions may occur through various mechanism, especially, inflammation, oxidative stress and disease formation [7].

Dicarbonyl methylglyoxal (MGO) is an RCS that is generated in diabetes mellitus and other metabolic conditions where carbonyl stress prevails. It acts as a potent glycating agent and is capable of reacting with proteins to form AGEs residues directly and rapidly, inducing cell and tissue damage [8]. For several decades, RCS and their reaction products have been considered as markers of oxidative stress, and this notion was based on the observation between

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the correlation of disease states and the amount of RCS and AGEs in tissue and fluid (both in animals and humans) [9]. As biomarkers, they represent a promising avenue for targeting compounds effective for the inhibition of AGEs and/or as sequestering agents that block their biological effects and significantly counteract different oxidative stress-based diseases.

Sulfur (thiol)-containing compounds have facile reactivity towards electrophilic compounds, making them good candidates for detoxifying carbonyl species [10], however, very few inhibitors have been identified to date. Moreover, evaluation of several sequestering models using analytical chemistry techniques can be costly and time-consuming. So, to evaluate thiol-containing small molecules, *in silico*-based approaches can be employed to better understand their reaction mechanism and their ability to sequester RCS – and, ultimately, their administration in the prevention of the development of oxidative-based diseases.

CYS is a thiol-containing molecule reported to have higher bioavailability in erythrocytes and is an established precursor to endogenous antioxidants such as glutathione (GSH) and could potentially modulate RCS- or ROS-induced toxicities causing deleterious cellular damage [11]. We hypothesize that thiol-containing molecules are promising therapeutic tools for scavenging or neutralizing the harmful and toxic effects of MGO or MGO-induced production of AGEs, thereby, preventing cellular or tissue dysfunctions. In this study, we evaluated antiglycation, the potential preventive effects towards protein damage and the ROS or RCS scavenging actions of CYS using muscle cells under metabolic stress. Currently, there is limited data to indicate the site selectivity or reactivity of CYS towards MGO, thus, this is an additional highlight of this study. Therefore, this combined approach will serve as a baseline to search and evaluate more related molecules with preventive effects against reactive carbonyl species-mediated protein damage.

MATERIALS AND METHODS

Materials

All chemicals or reagents were purchased from Sigma Aldrich (Science Park Drive, Singapore) through Chemline and Krypton Philippines. C2C12 cells were acquired from ATCC (Washington, USA) through ChemoLife Science, and cell culture reagents and plastic wares were purchased from ThermoFisher Scientific, through Noveulab and Medtest, Philippines.

DFT Studies

All calculations of the electronic structures of CYS and MGO were carried out in the gas phase (298.15 K and 1 atm), using the Gaussian-16 [12] software package at the DFT/6-31G(d) level of theory. Ground state structures were confirmed by the absence of an imaginary frequency in the Hessian. Optimized structures were then utilized as input files for global and local reactivity descriptor calculations by way of application of the Mutiwfn v.7 program [13]. The ionization potential (*I*) was calculated utilizing the equation:

$$I = E^{N-1} - E^{N}, \quad (1),$$

Electron affinity (*A*) then was ascertained via:

$$A = E^{N} - E^{N+1}, \quad (2),$$

where E^{N-1}, E^{N}, and E^{N+1} are the one-less-electron, neutral, and one-more-electron energies for each compound.

From these values, hardness (η) was calculated from the following equation:

$$\eta = (I-A)/2 \quad (3),$$

For the chemical potential (μ), the following equation was applied:

$$\mu = (A-I)/2 \quad (4),$$

Lastly, electrophilicity index (ω) values were obtained from hardness and chemical potential values by employing the following equation:

$$\omega = \mu^2/2\eta \quad (5).$$

Antiglycation Activity

BSA-MGO Model System

Bovine serum albumin (BSA) (20 mg/ml) was incubated for 7 days at 37°C with 60 mM methyl glyoxal (MGO) in 0.01 mM phosphate buffer (pH 7.4) in the presence or absence of CYS. The fluorescence intensity was subsequently recorded using a microplate reader (Clariostar, BMG Labtech, Germany) at 330 (emission) and 392 (excitation) wavelengths. Non-glycated BSA was employed as a control. The % inhibition of AGE formation in the test samples was expressed in terms of change relative to (non-glycated) control. This method was adapted from the published method [14].

BSA-Glucose Model System

The BSA-Glucose glycation model was applied to assess CYS as an anti-sequestering agent against AGE formation. This method was adapted from the published method [14]. A concentration of 90 mg/mL of glucose was allowed to react with BSA (10 mg/mL) in the presence or absence of CYS (1 mM). The fluorescence intensity was then recorded using a microplate reader (Clariostar, BMG Labtech, Germany) at 330 (emission) and 394 (excitation) wavelengths. Non-glycated BSA was employed as a control. The % inhibition of AGE formation in the test samples was expressed in terms of change relative to (non-glycated) control.

Congo Red Binding Assay

To preliminarily assess if CYS could potentially prevent protein tertiary structure modifications, a dye-based binding assay was utilized. This assay was performed based on a published method by Mehran *et al.* [15]. Briefly, three groups: non-glycated (BSA only), (BSA (10 mg/mL), and D-glucose (90 mg/mL) in the absence or presence of CYS,

were prepared. All solutions were dissolved in 0.2-M phosphate buffer saline (pH 7.4) containing 3.0 mM sodium azide. The final volume of the incubation was 1.8-mL. Glycated groups were incubated at 60°C for 1 day to accelerate the reaction. Non-glycated group was not incubated. After incubation, half of the samples were added to 0.5-mL Congo red solution (75 µM) in phosphate buffer saline-ethanol 10% (v/v). The other half was then used for background correction. Absorbance readings were recorded at 530 nm by a UV-Vis spectrophotometer. Similar assays were conducted this time using 60 mM MGO instead of glucose.

Cell Culture and Treatment

Mouse C2C12 skeletal myoblasts were maintained in high glucose-Dulbecco's Modofied Eagle Medium (DMEM) supplemented with 20% fetal bovine serum, 2% L-glutamine and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO^2 at 37°C. At desired confluency, the medium was switched to DMEM supplemented with 2% heat-inactivated horse serum for 7 days to facilitate myocytic differentiation. Cells were then incubated for a further 24 hours in the presence or absence of a glucolipotoxic (GLT) media (high-glucose (28-mM) and high fatty acids (200 uM oleic acid and palmitic acid) or in the presence or absence of 500-uM MGO before analysis as described below.

Reactive Species Detection

Muscle cells were cultured as described above in standard tissue culture media, or media supplemented with GLT media or MGO. CYS (1.0-mM) was then added and incubated for 1 hour. Cells were subsequently washed 3 times in DPBS, then 20 µM DCFDA loaded for 1 h. Radical species detection was measured via fluorescence, with excitation at 495 nm and emission at 530 nm. In all cases, ROS was expressed as a percentage change relative to control.

Cell Viability

C2C12 muscle cells were cultured in (+/-) GLT media or (+/-) MGO for 24-hrs. CYS (1.0-mM) was then added and incubated for 1 hour. Afterwards, the cells were washed 3 times in Dulbecco's Phosphate Buffer Saline (DPBS), then a 200-uL of filtered-sterilized resazurin solution (0.15 mg/mL) in growth media was added to each well and incubated for 1 hr. Cell viability was measured via fluorescence, with excitation and emission at 560 nm and 590 nm filter set, respectively.

DPPH Scavenging Activity

The free radical scavenging activity of CYS was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the method [16]. Briefly, a 500-uL of CYS (1 mM) was added to 1 mL solution of DPPH (0.1 mM) in methanol. The resulting solution was mixed thoroughly and allowed to react at room temperature in the dark for 30 minutes. The absorbance of the solution was then recorded at 517 nm. Vitamin C was utilized as the positive control and methanol as blank. The control contained only a methanolic solution of DPPH in the absence of CYS. Scavenging activity was subsequently estimated, and expressed in terms of percentage change relative to control.

Statistical analysis

All reported values are expressed as Mean ± SEM from 3 independent trials. A one-way analysis of variance was performed to determine whether or not there is significant difference among the groups. Where necessary, a post hoc analysis (Tukey's test) was performed and $p < 0.05$ was considered significant. Analysis were performed using GraphPad Prism ver. 10.2.0 (392).

RESULTS

Reactivity Analysis

This study demonstrates how CYS could potentially sequester the reactive MGO. In here, we report the global reactivity (Tab. 1) of CYS and MGO wherein five descriptors were calculated: the ionization potential (I), electron affinity (A), hardness (η, chemical potential (µ) and electrophilicity (ω). Global reactivity descriptors of GSH were used as reference, as the thiol compound is the main regulator of MGO in biological systems [17].

Table 1. Global Density Functional Theory Reactivity Descriptors (eV)

Global Descriptor	Compounds		
	MGO	CYS	GSH
I	9.68	8.78	8.07
A	0.04	-1.90	-1.28
η	4.82	5.34	4.67
µ	-4.86	-3.44	-3.40
ω	2.45	1.11	1.23

The local reactivity of each atom was also analyzed through the molecular orbitals, and the condensed Fukui function was applied to determine nucleophilic attack (f^+), electrophilic attack (f^-) and condensed dual descriptor (Δf). Figure 1 shows the frontier molecular orbital plots of CYS and methylglyoxal. The Fukui functions allowed identification of susceptible local areas to electrophilic (f^-) and nucleophilic (f^+) attacks, as well as assessment of the condensed dual descriptor (Δf). In Table 2, sulfur bears the largest value for (f^-), suggesting it is the most susceptible atom for an electrophilic attack, while a nucleophilic attack at carbon 1 of MGO is the most likely to occur: bearing the largest value of (f^+).

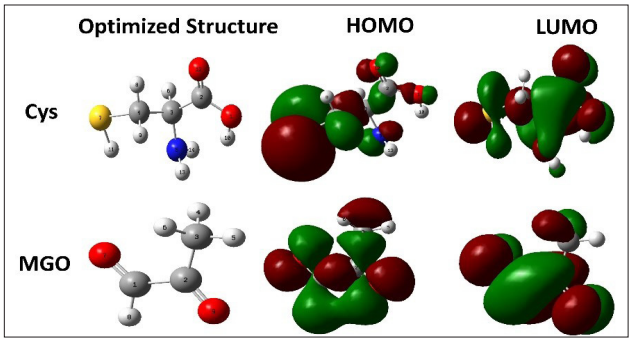


Figure 1. Frontier molecular orbitals of CYS and Methylglyoxal (DFT/B3LYP/6-31G (d))

Table 2. Condensed Fukui functions and dual descriptors of CYS and MGO

L-cysteine				Methylgloxal			
Atom	f^+	f^-	Δf	Atom	f^+	f^-	Δf
O1	0.0812	0.0676	0.0137	C1	0.1902	0.1145	0.0757
C2	0.1199	0.0486	0.0713	C2	0.1504	0.0979	0.0525
C3	0.0354	0.0285	0.0069	C3	0.0376	0.0672	-0.0297
C4	0.0393	0.0282	0.0111	H4	0.0424	0.0477	-0.0053
N5	0.0388	0.0824	-0.0437	H5	0.0445	0.0485	-0.0040
H6	0.0582	0.0253	0.0329	H6	0.0424	0.0477	-0.0053
S7	0.2138	0.3749	-0.1611	O7	0.2101	0.2251	-0.0150
H8	0.0284	0.0271	0.0013	H8	0.0856	0.1013	-0.0157
H9	0.0398	0.0364	0.0033	O9	0.1968	0.2500	-0.0532
H10	0.0457	0.0251	0.0206				
H11	0.0523	0.0518	0.0004				
O12	0.1340	0.1313	0.0027				
H13	0.0536	0.0341	0.0195				
H14	0.0597	0.0388	0.0209				

Congo Red Assay

Based on the computational data obtained above, we sought to validate this observation by doing *in vitro* analysis. First, the effect of MGO on potential protein structural damage was investigated using Congo Red binding assay. After removing the background, it can be seen in Table 3 that Congo red absorbance was significantly increased in both glycated conditions, as compared to native non-glycated BSA. Importantly, CYS demonstrated significant protection against protein structural alteration.

Table 3. Effects of CYS on Secondary Structural Alteration of BSA (% Change Relative to Control Non-glycated)

Non-glycated	Glycated	L-cysteine	Aminoguanidine
Congo-Methylglyoxal Model System			
100.00	186.68 (2.51) *	168.88 (5.45) #	136.57 (3.85) #
Congo-Glucose Model System			
100.00	169.50 (21.11) *	100.34 (6.64) #	152.77 (30.50)

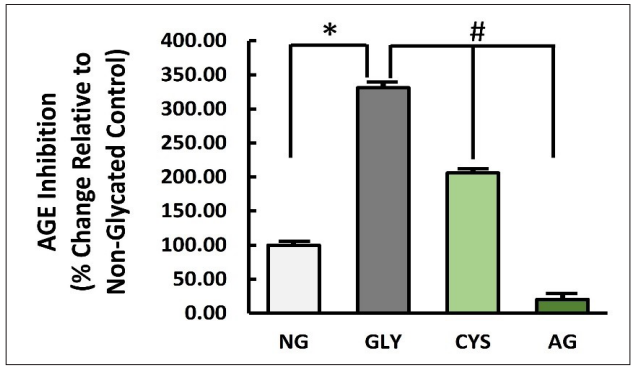
* p<0.05 wrt Non-glycated; # p<0.05 wrt Glycated; Tukey's test

Results expressed as mean (SEM) from n=3 independent trials

Antiglycation Activity

We sought to determine whether or not the reactivity of CYS based on the theoretical data described above will have an antiglycation effect. Based on Figures 2 and 3, it can be seen that methylglyoxal (3.31 times higher) and glucose (1.90 times higher) resulted in a significantly increased glycation or AGE formation, as compared to non-glycated BSA. Interestingly, the addition of CYS significantly ($p < 0.01$) sequestered this MGO-induced formation of AGE, and, importantly, reversed this glycation event – as depicted in the BSA-glucose model system.

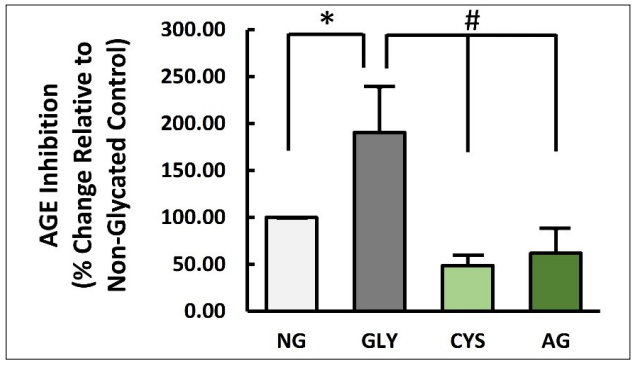
In Figure 4, a 24-hour exposure of cells to glucolipotoxic condition or (GLT) media increased the reactive species significantly by 19.60±1.36%, as compared to the healthy control. Also, under similar treatment conditions, we observed a similar effect with regard to MGO-treated cells. Although there were no observed significant scavenging effects of CYS towards GLT-induced reactive oxygen (ROS) formation, we noted that a 1-hour dose of 1 mM CYS significantly ($p < 0.01$) sequestered the reactive aldehyde MGO. This observation from the latter could be ascribed to the specific reactivity of CYS towards reactive aldehydes such as methylglyoxal.



Mean ± sem; p<0.01; * compared to NG; # compared to glycated (GLY); Tukey's test

Data are expressed as percentage change relative to non-glycated (NG) control from n=3 independent trials

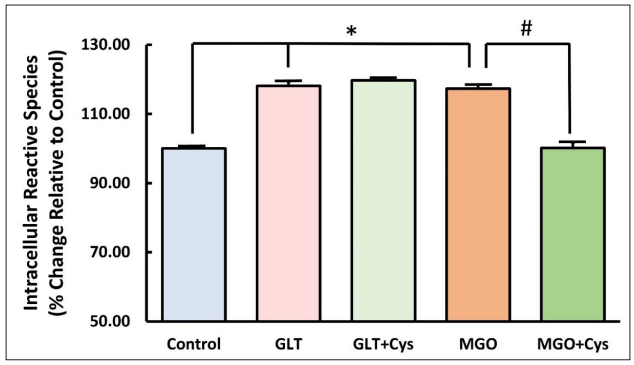
Figure 2. CYS effectively sequesters and prevents methylglyoxal-induced glycation of BSA



Mean ± sem; p<0.05; * compared to NG; # compared to glycated (GLY); Tukey's test

Data are expressed as percentage change relative to non-glycated (NG) control from n=3 independent trials

Figure 3. BSA glycation with glucose increased potential AGE formation and is significantly inhibited by CYS



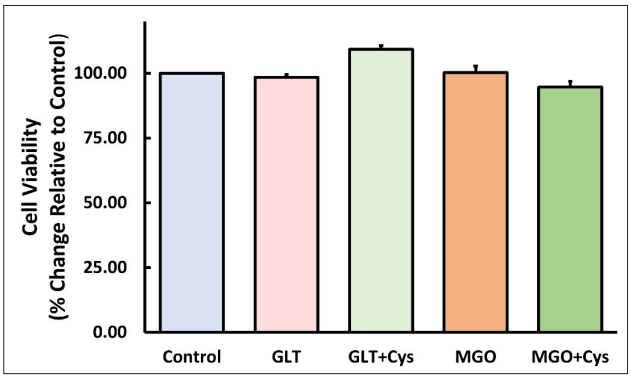
Mean ± sem; p<0.01; * compared to Control; # compared to methylglyoxal-treated cells (MGO); Tukey's test

Data are expressed as percentage change relative to Control

Figure 4. CYS is an effective sequestering agent of methylglyoxal

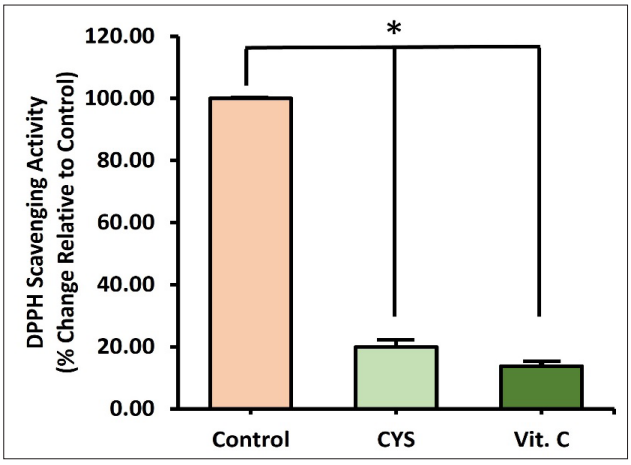
In order to determine whether or not treatment conditions could affect significantly the cell viability, a resazurin-based cell viability was conducted. In Figure 5, since there

was no significant difference obtained among the groups, it can be inferred that the level of ROS generated and the extent of glycation observed with MGO and GLT, and the protection exhibited by CYS is not due to cell death or cell proliferation.



Mean ± sem; p>0.05; ANOVA
Data are expressed as percentage change relative to healthy control

Figure 5. CYS, GLT, and MGO treatment to cells did not affect cell viability



Mean ± sem; * p<0.01; Tukey's Test

Figure 6. CYS is an effective scavenger of free-radical species. Data are expressed as percentage change relative to control

DISCUSSION

Computational analysis in this study provides initial insights as to which part of our target molecules most likely are involved in the desired reaction. Data in Table 1 indicates that CYS exhibited a lower ionization potential compared to GSH. This means that CYS needed higher energy to lose an electron than GSH. On the contrary, MGO obtained a value of 0.04 eV (the highest among the three), implying that this compound possessed a good chance of accepting an electron. These outcomes suggest that CYS should be an electron donor, while MGO should be an electron acceptor in their reaction. Also, values calculated from the electrophilicity index (ω) support the notion that MGO is the most electrophilic compound (2.45 eV), while CYS is the least electrophilic compound (1.11 eV). Overall, the global descriptor data confirms CYS to be a good sequestering agent against MGO. In Figure 1 and Table 2, data indicated that the most interesting orbitals were the MGO-LUMO located along the carbonyl carbon groups and the CYS HOMO orbital located on the sulfur atom of the thiol group, and thus allowed us to identify susceptible local areas to electrophilic (f^-), nucleophilic (f^+) attacks, and to determine the condensed dual descriptor (Δf). Padmanabhan *et al.* [18] propose that when $\Delta f > 0$, the process is driven by a nucleophilic attack on that atom, and that atom acts as an electrophilic species. Conversely, when $\neg\ \Delta f < 0$, the process is driven by an electrophilic attack over that atom and therefore acts as a nucleophilic species. Thus, electrophilic attack at carbon 1 of methylglyoxal and nucleophilic attack by sulfur of CYS are all supported by their respective condensed dual descriptor values of 0.0757 and -0.1611, respectively.

Glycation events brought by AGEs and its precursors towards BSA have been reported [19]. Congo red can be utilized to assess this as it has a specific binding affinity to beta-pleated sheets conformation in amyloid-like peptides, a formation of which exhibits a specific absorption at 530 nm post binding [20]. In this assay, we could preliminarily assess how glycation harms protein folding. When the MGO and the glucose were allowed to react with native BSA, stress induced changes in the structure of the protein (as seen in the change of absorbance). The deleterious consequences of AGEs are demonstrated in several diseases, including diabetes [21], chronic kidney disease [22], cardiovascular diseases [23] and Alzheimer's disease [24]. As indicated above, CYS could potentially protect protein against structural alteration possibly induced by reactive species glycation, which means that CYS is a potential good candidate molecule for use as an antiglycation agent. Therefore, any molecule like CYS that could prevent RCS-induced protein glycation might provide beneficial action toward prevention (if not inhibition) of the development of some debilitating disease progression or complications.

Based on the potential action demonstrated by the thiol-containing CYS as shown above, we next investigated the beneficial action this molecule has upon GLT and MGO-treated mouse C2C12 cells as a cellular model of oxidative stress. In order to determine the level of cellular reactive species generated from each treatment condition, a fluorescence-based assay was employed using a cell-permeant fluorogenic dye called 2'7'-dichlorofluorescein diacetate (DCFDA) which is deacetylated by cellular esterases, and, upon the presence of oxidizing species, is converted into 2',7'-dichlorofluorescein (DCF) – a highly fluorescent compound that can be detected by fluorescence spectroscopy. It was reported previously in the study [25] that exposure of the C2C12 cells for up to 5 days in glt media results in about 75% increase in ROS generation. This and our present study add to the growing evidence of how oxidative stress (OS) is attributed as a common denominator for the pathogenesis of several diseases, including cancer, diabetes, obesity and neurodegenerative disorders (among others) [26-28].

OS ensues when oxidant product in the living system exceeds that of the cell's antioxidant machinery, in other words, a disease results when there is an imbalance of the redox system of the cell. CYS is a promising molecular tool that could potentially increase, if not, sustain the endogenous pool of antioxidants in the human body, thereby enhancing cellular response towards oxidative stress. The detection

of ROS activity or cellular reactive species detection was not normalized to either cell number or protein content and so it was necessary to investigate the effect of GLT on cell number and viability to ensure that the response shown in GLT was not an artifact of either glucolipotoxic- or methylglyoxal-driven cell death or glucose- or methylglyoxal driven cell proliferation. A resazurin-based cell viability [29] was performed to determine cellular metabolic activity and the mitochondrial viability of live cells. The dye indicator (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) measures the redox reactions that primarily occur in the live cells, and metabolically active cells will turn the blue non-fluorescent agent into a highly fluorescent pink resorufin. Since there was a non-statistically significant change observed in the cell viability and number of cells exposed to either GLT or MGO after 24 hours with respect to control (Figure 5), it can be inferred that the significant change detected in the level of reactive species above as depicted in Figure 4 were not due to C2C12 cell viability. Reactivity of the thiol group in CYS towards radicals has also been demonstrated (Figure 6), and CYS was observed to be an effective scavenger (80.09±2.8%) against DPPH. The protective effect of CYS towards harmful free radicals could potentially benefit the cells and tissues that are negatively affected by oxidative stress.

CONCLUSION

The present study has shown evidential data from reactivity analysis based on global descriptors to indicate that CYS matches electron-donating capacity with the good electron acceptor, methylglyoxal. This pairing supports the feasible reaction of both agents and thus implies that the former is a good candidate molecule to be utilized as a sequestering agent against the latter. Furthermore, CYS has been shown to prevent protein structural modifications, which, in this case, may have a beneficial effect on protein folding-associated disease pathogenesis (although more mechanistic studies warrant this hypothesis). The ability of CYS to inhibit the formation of deleterious AGEs makes this thiol-containing compound a promising therapeutic tool for several AGEs-induced pathological conditions. The use of more relevant cellular and animal models of desired disease studies is recommended to better understand the mechanistic basis of CYS potential therapeutic effect. In addition, evaluating what effects CYS might have upon enzymes such as glyoxalase 1 and 2 (GLO 1 and 2) and aldose reductase (AR) that detoxify or convert MGO into a more stable form, is suggested. In this way, we can extend the study to discover and design more molecules that could benefit disease conditions exacerbated by RCS - such as MGO. Lastly, this study, utilizing the combined approach of computational and *in vitro* analysis, provides helpful data to maximize the potential impact of CYS, by designing related molecules that could serve as novel treatment strategies that are effective, safe and accessible to all in the future.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship, or otherwise, that could affect the research and its results presented in this article.

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