Supplementary Material

Harnessing ROS-induced oxidative stress for halting colorectal cancer *via* thiazolidinedione-based SOD inhibitors

Mohamed Nabil Abd Al Moaty *a*, El Sayed H. El Ashry *a*, Laila Fathy Awad *a*, Asmaa Mostafa *a*, Marwa M. Abu-Serie *b*, and Mohamed Teleb *c*

*a* Chemistry Department, Faculty of Science, Alexandria University, Alexandria 21321, Egypt.

*b* Medical Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications (SRTA-City), Egypt.

*c* Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Alexandria University, Alexandria 21521, Egypt.

* Corresponding author; Email address: laila.fathy@yahoo.com

| Content                                                                 | Page   |
|------------------------------------------------------------------------|--------|
| 1. NMR spectra of compounds 4-7                                         | S2-S17 |
| 2. Biological evaluation                                               |        |
| 2.1. Cytotoxicity screening on normal human cells                       | S18    |
| 2.2. Anticancer screening                                              | S18    |
| 2.3. SOD inhibition assay                                              | S18    |
| 2.4. Inhibition of the antioxidant enzymes in colorectal cancer cells   | S19    |
| (Caco-2)                                                                |        |
| 2.5. Determination of cellular reactive oxygen species in colorectal   | S19    |
| cancer (Caco-2)                                                        |        |
| 3. Docking simulations                                                 | S19    |
| References                                                             | S20    |
Figure S1. $^1$H-NMR (CDCl$_3$) spectrum of 3-Allyl-5-benzylidene-2,4-thiazolidinedione 4a.

Figure S2. $^{13}$C-NMR (CDCl$_3$) spectrum of 3-Allyl-5-benzylidene-2,4-thiazolidinedione 4a.
**Figure S3.** $^1$H-NMR (CDCl$_3$) spectrum of 3-Allyl-5-(3-chlorobenzylidene)-2,4-thiazolidinedione 4b.

**Figure S4.** $^{13}$C-NMR (CDCl$_3$) spectrum of 3-Allyl-5-(3-chlorobenzylidene)-2,4-thiazolidinedione 4b.
Figure S5. $^1$H-NMR (CDCl$_3$) spectrum of 3-Allyl-5-(4-nitrobenzylidene)-2,4-thiazolidenedione 4c.

Figure S6. $^{13}$C-NMR (CDCl$_3$) spectrum of 3-Allyl-5-(4-nitrobenzylidene)-2,4-thiazolidenedione 4c.
Figure S7. $^1$H-NMR (CDCl$_3$) spectrum of 3-Allyl-5-(4-dimethylaminobenzylidene)-2,4-thiazolidenedione 4d.

Figure S8. $^{13}$C-NMR (CDCl$_3$) spectrum of 3-Allyl-5-(4-dimethylaminobenzylidene)-2,4-thiazolidenedione 4d.
Figure S9. $^1$H-NMR (CDCl$_3$) spectrum of 3-Allyl-5-(4-allyloxy)benzylidene)-2,4-thiazolidenedione 4e.

Figure S10. $^{13}$C-NMR (CDCl$_3$) spectrum of 3-Allyl-5-(4-allyloxy)benzylidene)-2,4-thiazolidenedione 4e.
Figure S11. $^1$H-NMR (CDCl$_3$) spectrum of Ethyl-2-(5-benzylidene-2,4-dioxothiazolidin-3-yl)acetate 5a.

Figure S12. $^{13}$C-NMR (CDCl$_3$) spectrum Ethyl-2-(5-benzylidene-2,4-dioxothiazolidin-3-yl)acetate 5a.
Figure S13. $^1$H-NMR (CDCl$_3$) spectrum of Ethyl-2-(5-(4-nitrobenzylidene)-2,4-dioxothiazolidin-3-yl)acetate 5c.

Figure S14. $^{13}$C-NMR (CDCl$_3$) spectrum Ethyl-2-(5-(4-nitrobenzylidene)-2,4-dioxothiazolidin-3-yl)acetate 5c.
**Figure S15.** $^1$H-NMR (CDCl$_3$) spectrum of Ethyl-2-(5-(4-dimethylaminobenzylidene)-2,4-dioxothiazolidin-3-yl)acetate 5d.

**Figure S16.** $^{13}$C-NMR (CDCl$_3$) spectrum Ethyl-2-(5-(4-dimethylaminobenzylidene) -2,4-dioxothiazolidin-3-yl)acetate 5d.
Figure S17. $^1$H-NMR (CDCl$_3$) spectrum of Ethyl-2-(5-(4-(2-ethoxy-2-oxoethoxy)benzylidene)-2,4-dioxothiazolidin-3-yl)acetate 5e.

Figure S18. $^{13}$C-NMR (CDCl$_3$) spectrum of Ethyl-2-(5-(4-(2-ethoxy-2-oxoethoxy)benzylidene)-2,4-dioxothiazolidin-3-yl)acetate 5e.
Figure S19. $^1$H-NMR (CDCl$_3$) spectrum of 5-benzylidene-3-(3-bromopropyl)-2,4-thiazolidinedione 6a.

Figure S20. $^{13}$C-NMR (CDCl$_3$) spectrum 5-benzylidene-3-(3-bromopropyl)-2,4-thiazolidinedione 6a.
Figure S21. $^1$H-NMR (CDCl$_3$) spectrum of N-(3-bromopropyl)-5-(4-nitrobenzylidene)-2,4-thiazolidinedione 6c.

Figure S22. $^{13}$C-NMR (CDCl$_3$) spectrum of N-(3-bromopropyl)-5-(4-nitrobenzylidene)-2,4-thiazolidinedione 6c.
**Figure S23.** $^1$H-NMR (CDCl$_3$) spectrum of 3-(3-bromopropyl)-5-(4 dimethylaminobenzylidene)-2,4-thiazolidinedione 6d.

**Figure S24.** $^{13}$C-NMR (DMSO-d$_6$) spectrum of 3-(3-bromopropyl)-5-(4-dimethylaminobenzylidene)-2,4-thiazolidine-dione 6d.
Figure S25. $^1$H-NMR (CDCl$_3$) spectrum of 5-(4-(3-bromopropoxy)benzylidene)-3-(3-bromopropyl)-2,4-thiazolidinedione 6e.

Figure S26. $^{13}$C-NMR (CDCl$_3$) spectrum of 5-(4-(3-bromopropoxy)benzylidene)-3-(3-bromopropyl)-2,4-thiazolidinedione 6e.
Figure S27. $^1$H-NMR (CDCl$_3$) spectrum of 5-benzylidene-3-(3-hydroxypropyl)-2,4-thiazolidinedione 7a.

Figure S28. $^{13}$C-NMR (CDCl$_3$) spectrum of 5-benzylidene-3-(3-hydroxypropyl)-2,4-thiazolidinedione 7a.
Figure S29. $^1$H-NMR (CDCl$_3$) spectrum of 3-(3-hydroxypropyl)-5-(4-nitrobenzylidene)-2,4-thiazolidinedione 7c.

Figure S30. $^{13}$C-NMR (CDCl$_3$) spectrum of 3-(3-hydroxypropyl)-5-(4-nitrobenzylidene)-2,4-thiazolidinedione 7c.
Figure S31. $^1$H-NMR (CDCl$_3$) spectrum of 5-(4 dimethylaminobenzylidene)-3-(3-hydroxypropyl)-2,4-thiazolidinedione 7d.

Figure S32. $^{13}$C-NMR (CDCl$_3$) spectrum of 5-(4 dimethylaminobenzylidene)-3-(3-hydroxypropyl)-2,4-thiazolidinedione 7d.
2. Biological evaluation

2.1. Cytotoxicity screening on normal human cells

Wi-38 cell line was cultured in DMEM medium-contained 10% fetal bovine serum (FBS), seeded as $5 \times 10^3$ cells per well in 96-well cell culture plate and incubated at 37°C in 5% CO₂ incubator. After 24 h for cell attachment, serial concentrations of the synthetic compounds and doxorubicin (Dox “as standard chemotherapy drug”) were incubated with Wi-38 cells for 72 h. Cell viability was assayed by MTT method. Twenty microliters of 5 mg/ml MTT (Sigma, USA) was added to each well and the plate was incubated at 37°C for 3 h. Then MTT solution was removed, 100 µl DMSO was added and the absorbance of each well was measured with a microplate reader (BMG LabTech, Germany) at 570 nm. The effective safe concentration (EC₁₀₀) value (at 100% cell viability) of the tested compounds was estimated by the Graphpad Instat software.

2.2. Anticancer screening

Anticancer effect of the studied compounds was assayed using human colon cancer cell line Caco-2. Caco-2 was cultured in DMEM (Lonza, USA) supplemented with 10% FBS. All cancer cells ($4 \times 10^3$ cells/well) were seeded in sterile 96-well plates. After 24h, serial concentrations of the tested compounds and Dox were incubated with three cancer cell lines for 72 h at 37°C in 5% CO₂ incubator. MTT method was done as described above. The half maximal inhibitory concentration (IC₅₀) values were calculated using the Graphpad Instat software. Furthermore, cellular morphological changes before and after treatment with the most effective and safest anticancer compounds were investigated using phase contrast inverted microscope with a digital camera (Olympus, Japan).

2.3. SOD inhibition assay

HeLa cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂with RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (1×). The mitochondrial fraction can be separated by using the Mitochondria/Cytosol Fractionation Kit (Catalog Number MIT1000, Merck KGaA, Darmstadt, Germany). The superoxide dismutase activity in HeLa cell lysates was determined by using the superoxide dismutase kit from R&D Systems according to the manufacturer’s instructions. In the assay, superoxide anions generated by xanthine oxidase (XOD)convert nitrobluetetrazolium (NBT) to NBT-diformazan which absorbs light at 550 nm. SOD reduces the superoxide anionconcentration and thereby lowers the rate of NBT-diformazanformation. Briefly, cells were detached with trypsin after treatment and washed once with cold PBS. The cell pellet was suspended in cell lysis solution at $5 \times 10^6$ cells/ml. Cell lysates were centrifuged at 14,000g for 5 min at 4°C and supernatants were kept on ice. For each assay, 500 µl of the supernatants was
mixed with xanthine, NBT, and XOD solutions and absorbance at 550 nm was measured 5 min after the beginning of reaction.

We determined the 100% of SOD activity by measuring the difference between the absorbances of HeLa control cells and a negative control without cell lysate.

Percentage inhibition was calculated using this equation:

\[
\% \text{ superoxide dismutase inhibition} = \left[ \frac{\text{normal activity} - \text{inhibited activity}}{\text{normal activity}} \right] \times 100%.
\]

**2.4. Inhibition of the antioxidant enzymes in colorectal cancer cells (Caco-2)**

These antioxidant enzymes include aldehyde dehydrogenase (ALDH) 1A, glutathione peroxidase (GPX) and superoxide dismutase (SOD). The ALDH1A activity was detected according to Graham et al \(^1\) using all-trans-retinal as substrate. The cell lysate was incubated with 500 µM NAD\(^+\), as ALDH cofactor, for 10 min at 37°C. The substrate was then added to initiate ALDH reaction. The absorbance of NADH generation/ min at 340 nm was measured using microplate spectrophotometer (BMG LabTech, Germany). The GPX activity was determined using the methods described by Rotruck et al.\(^2\) Briefly, GPX (Sigma, USA) was incubated at 37°C with cumene hydroperoxide and the reduced glutathione (GSH) as substrates. The enzyme was not used as a control. After 10 min, Ellman’s reagent (19.8 mg% 5,5'-dithiobis-2-nitrobenzoic acid in 1% sodium citrate) was added and the absorbance of the developed yellow color was measured against blank at 412 nm. The activity of Cu/Zn SOD was measured using pyrogallol autooxidation method.\(^3\) The reaction mixture of 20 mM Tris-HCl buffer (pH 8.2) containing 20 mM pyrogallol and 1 mM diethylenetriaminopenta acetic acid was mixed with cell lysate. The change in absorbance during 2 min was measured at 420 nm.

**2.5. Determination of cellular reactive oxygen species in colorectal cancer (Caco-2)**

The cellular reactive oxygen species (ROS) was determined by incubating the untreated and the most effective anticancer compounds-treated Caco-2 with 2’,7’-dichlorofluorescin diacetate (DCFDA). DCFDA is oxidized by cellular reactive oxygen species (ROS) to fluorescent 2’, 7’-dichlorofluorescein (DCF), which was measured with fluorescence spectroscopy (BMG LabTech, Germany) at 480 nm excitation and 530 nm emission.

**3. Docking**

Docking simulations were performed employing Molecular Operating Environment (MOE) software package version 2015.10,\(^4\) Chemical Computing Group, Montreal, Canada. SOD crystal structure \(^5\) was obtained from the protein data bank, prepared via the default MOE structure preparation settings. Energy minimization and geometry optimization of the studied TZDs were done through the MOE dock tool prior to docking. The prepared ligands were located in the
active site near the Zn domain then various docking protocols were employed. Best results were obtained utilizing rigid docking protocol. The ligand placement method was set to apply the Triangular matcher algorithm. Alpha HB scoring function was employed as the default scoring function generating the top 10 non-redundant poses of the lowest binding energy conformers of the test ligands.

**References**

1. Graham C.E.; Brocklehurst K.; Pickergill R.W. and Warren M.J. Characterization of retinaldehyde dehydrogenase 3. *Biochem. 2006*, **394**(1), 67-75.
2. Rotruck J.T.; Pope A.L.; Ganther H.E.; Swanson A.B.; Hafeman D.G. and Hoekstra W.G. Selenium: biochemical role as a component of glutathione peroxidase. *Science 1973*, **179**(4073), 588-590.
3. Marklund S. and Marklund G. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem. 1974*, **47**, 469-474.
4. C.C.G. Molecular Operating Environment (MOE), Montreal, Canada, [http://www.chemcomp.com](http://www.chemcomp.com)
5. Sala, F.A.; Wright, G.S.A.; Antonyuk, S.V.; Garratt, R.C. and Hasnain, S.S._Molecular recognition and maturation of SOD1 by its evolutionarily destabilized cognate chaperone hCCS._ *PLoS Biol. 2019*, **17**, e3000141-e3000141.