Melatonin reduces cell motility and antioxidant defenses in ovarian cancer cell lines

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Research Article

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Abstract

Ovarian cancer (OC), a highly recurrent and fatal tumor, poses diagnostic challenges due to generic symptoms and chemoresistance. Melatonin (Mel) is an indoleamine acting against tumor progression and exhibiting pro-oxidative actions in tumor cells. This study explores the impact of Mel on antioxidant defenses of OC cells (SKOV-3 and CAISMOV-24 lines), focusing on its receptor-dependent and -independent effects. Cell viability was assessed using the MTT method and the antioxidant system was analyzed by preparing supernatants for assessing glutathione (GS), reduced glutathione (GSH), oxidized glutathione (GSSG), catalase (CAT), glutathione S-transferase (GST), and superoxide dismutase (SOD). Mel stimulated its own intracellular levels, reducing cell viability in both cell lines. Notably, Mel independently of its membrane receptors, inhibited migration and invasion, thus showing its anti-tumoral potential. By investigating melatonin's actions, we observed an impact on the antioxidant system primarily through the reduced activity of CAT and the GS axis. The modulation of these antioxidants by Mel demonstrates its multifaceted role in OC, emphasizing its therapeutic potential. We also demonstrated, for the first time, the theoretical ability of Mel to bind to CAT, which may be responsible for the reduction in enzyme activity. This study contributes with novel insights into Mel's receptor-independent actions, providing a foundation for further research in OC therapy.

Introduction

Ovarian cancer (OC) is one of the most common female tumor subtypes which has a high recurrence and mortality rate [1]. This mortality rate is associated with its late diagnosis due to multiple symptoms that are often generic and their aggressive recurrence even with already established treatments and surgical removal [2, 3]. Modifications in cellular metabolism occur when tumors redefine how they obtain nutrients and navigate metabolic routes to fulfill the requirements for biological energy, duplication, and the control of reactive oxygen species (ROS) levels in the cancer cells examined [4]. These changes are recognized as key aspects of cancer metabolism, marked by features such as tissue invasion and metastasis, loss of growth inhibition, rapid proliferation, sustained cell viability, resistance against cell death, initiation of the angiogenesis pathway, evasion of the immune response, and modified cellular vitality [5, 6].

Melatonin (Mel; N-acetyl-5-methoxytryptamine) is a lipophilic molecule converted from serotonin by the enzymes arylalkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT) [7]. Mel has been characterized by actions that may either depend on or be independent of its membrane receptors (MT1 and MT2). While its main production occurs in the pineal gland at night, other reports have shown its synthesis in different tissues and cells with local actions [8]. Mel has numerous functions under normal metabolic conditions including its antioxidant actions by removing reactive oxygen and nitrogen species and stimulating DNA repair mechanisms [9]. In many cancer cell types, Mel has oncostatic functions associated with pro-oxidative function while concurrently reducing the migration and invasiveness of cancer cells and inducing apoptosis. [10, 11].

Free radicals (FR) are a generic term that encompasses unstable and reactive atoms or molecules. In biological systems, FR can be separated into two classes: reactive oxygen species (ROS) and reactive nitrogen species (RNS) [12]. Superoxide anion (O2•−), hydroxyl radical (OH•), alkoxy radical (RO•), hydrogen peroxide (H_2O_2), and hypochlorous acid are considered ROS and they are normally generated during cellular metabolism; nitrogen dioxide (NO_2), nitroxyl (HNO) and peroxynitrite (ONOO-) are examples of RNS produced from nitric oxide and by the NADPH oxidase (NOX) system [12]. In healthy cells, there is a delicate balance between the production and removal of FR, which ensures redox homeostasis and their optimal physiological effects [13].

FR affects gene expression, cell growth and differentiation, modulate metabolic reactions, and regulate transcription factor activation, in addition to acting as intra and intercellular signaling molecules [14–17]. In OC cell lines, there is initially a significant increase in ROS production, thus favoring tumorigenesis. This elevated ROS results from NADPH oxidase activity and mitochondrial metabolism, as this increase is diminished by mitochondrial complex I inhibitors [18]. Elevated ROS which occurs at the level of the electron transport chain (ETC) is associated with chemoresistance to platinum-derived therapies leading to a poor prognosis and accelerating OC growth [19]. In addition, the increase in ROS is associated with dysregulation of microRNAs (miRNAs) which are linked to tumor progression. In serous epithelial OC, there is overexpression of specific miRNAs, an action that does not occur in the non-serous OC [20–23].

To avoid the damage caused by ROS, cells have antioxidant systems which are either enzymatic or non-enzymatic in nature. Among the main anti-oxidative enzymes are superoxide dismutase (SOD), catalase (CAT,), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), among others. It is important to consider that a specific antioxidant, to be efficient, does not necessarily need to be ubiquitous, but must act at the diffusion rate of the FR and be in the proper position in the immediate vicinity of where the radical is generated [24]. With the exception of H_2O_2, FR do not cross biological barriers and have a very short half-life. Mel is an antioxidant under normal circumstances but can serve a pro-oxidant under specific pathologies [25]. Although additional investigation is needed, Mel has been documented to promote an increase in ROS via different mechanisms [26, 27]. In head and neck cancer cells, Mel induced ROS by reversing mitochondrial ETC which generated the anti-growth behavior [28]. Mel also induces apoptosis mediated by the stimulation of ROS synthesis in breast cancer as well as in mesangial cells, thus documenting that the anti-cancer actions of melatonin may be mediated by excessive FR generation effects [26, 29, 30]. To date, the role of Mel on oxidative processes in OC have not been examined. Herein, we investigated the impact of Mel, acting via its membrane receptors or not, on antioxidant defenses of OC cells as well as its protection against cell migration and invasiveness.

Materials and Methods

Cell culture and reagents

SKOV-3 cell line (ATCC® HTB-77) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), while the CAISMOV-24 cell line was obtained from the Women's Hospital Prof. Dr.

José Aristodemo Pinotti Caism (UNICAMP, Campinas, SP, BRAZIL). SKOV-3 cells were cultivated using RPMI medium (Gibco, Paisley, UK), whereas CAISMOV-24 cells were nurtured in DMEN HAN'S F-12 (LGC, Cotia, BR). Both cell types received 10% fetal bovine serum (FBS) (Gibco) and penicillin at 100 IU/ml, as well as streptomycin at 100 µg/ml (Gibco). The cells were placed in a humidified environment at 37°C with 5% CO_2. Cellular expansion was carried out in distinct 75 and 25 cm^2 culture flasks (Costar, Cambridge, MA, USA). Upon achieving 80% of confluence, the cell supernatant was carefully extracted. Afterwards, the cells underwent two washes with 10% phosphate-buffered saline (PBS; Oxoid Limited, Hampshire, UK). Subsequently, trypsin/EDTA (Gibco) was employed to interrupt adhesion to the flasks.

Experimental design and treatments

To examine the effects of Mel and its combination with luzindole (Luz) on OC cells, we first determined the safe concentrations according to the IC_{50} method (MTT assay). Concentrations of 3.4 µM and 7 µM of Mel were chosen respectively to treat SKOV-3 and CAISMOV-24 cells for a period of 24h. The concentration of Luz (a Mel receptor antagonist) was defined as 1 µM for both cell lines. To understand the effect of Mel in combination with Luz on cancer cells, we divided each cancer cell line into three experimental groups: Control: cells exposed only to the culture medium containing 100 µL of DMSO solution as the vehicle; Mel: cells exposed to Mel at concentrations of 3.4 and 7 µM for SKOV-3 and CAISMOV-24, respectively, plus vehicle; and Mel + Luz: cells exposed to the combination of Mel and Luz plus vehicle. For the combination group, Luz was first administered, and after 30 min, the pre-established concentration of Mel was added. Mel and Luz were dissolved in DMSO (5%) maintaining the molarity values indicated by the manufacturers. All experimental assays were performed in biological and technical triplicates.

Cell viability (MTT assay)

Cell viability was analyzed using the MTT method based on the IC_{50} to determine proper Mel concentrations. After the confluence rate, SKOV-3 and CAISMOV-24 cells were trypsinized, seeded in 6-well plates at a density of $5x10^5$ cells per well, and cultured with appropriated medium supplemented with 10% FBS. After cell adherence, Mel and Luz were added to culture medium following the experiment design with specific concentrations (2.0, 3.4, 5.0, and 7.0 µM). Viability curves were estimated after 24h of treatment using the MTT solution (5mg/mL). To detect cytotoxicity, the reactions were replicated in 96-wells plate and read in a microplate reader (Epoch, Bio Tek Instruments, USA).

Cell invasion and migration assays

The evaluation of SKOV-3 and CAISMOV-24 cell invasiveness was performed using 24-well plates. A thin membrane of Geltrex® was added to each well, occluding the lower polyethylene terephthalate (PET) membrane. SKOV-3 and CAISMOV-24 ($1x10^5$ cells) were added to the top of the insert and received

standard medium without FBS. The invasive potential was analyzed based on the ability of cells to cross the gel barrier and the PET membrane through the pores, being attracted chemotactically by inferior coverage of culture medium containing 5% FBS. The plates were placed in a CO_2 atmosphere at 37ºC for 24h. After the incubation period, cells were fixed in methanol for 10 min, and the remaining cells were removed by scraping. Migrated cells were stained with a 0.1% toluidine blue solution and photographed with a 5X objective in an inverted microscope (ZeissAxiovert®). For migration assay, a similar experimental procedure was used, except for Geltrex® which was not added to the transwell chamber. All experiments were performed in triplicate based on four fields and submitted to automatic cell count LUNA-II® (Logos Biosystems, South Korea)

Measurement of melatonin concentration

Mel levels were determined in both SKOV-3 and CAISMOV-24 cells using a human-specific commercial ELISA kit (EH3344, Fine Test), according to manufacturer's instructions. The absorbance was read at 450 nm on a microplate reader (Epoch, Bio Tek Instruments, USA). Results were interpolated from standard curves generated by plotting the concentration of the standards against their absorbance. The concentrations are presented in pg/mL.

Preparation of the supernatant for analyzing the antioxidant system

After Mel treatment, the culture medium was discarded, and PBS was added to the cells. The cells were then lysed through three cycles of freezing (-80 ºC) and thawing (37 ºC) for 30 min each. After this process, protein levels were quantified and used to normalize the results of the total (GS), reduced (GSH), and oxidized glutathione (GSSG), catalase (CAT), glutathione S-transferase (GST), and superoxide dismutase (SOD).

Activity of SOD and CAT

After treatment with Mel and Luz, the cells (5 x 10^5 cells/mL) were washed with PBS (pH 7.4) and re-plated for all the analyzes. SOD enzyme activity assay was performed according to [31] with some modifications. The reaction medium was composed of methionine (13 mM), NBT (75 µM), and riboflavin (4 mM) in phosphate buffer (50 mM, pH 7.8). 10 µL of the cell extract was added, and the samples were exposed to fluorescent light (13W) for 5 min. Control samples (no enzymes) and the blank (containing all reagents but not exposed to light) were used. Readings were performed using a MultiSkan-Skyhigh microplate reader at 560 nm and at 25°C. For the evaluation of CAT activity, cells were lysed, and a reaction medium was prepared as described by Aebi et al. [32]. The reading was performed by a spectrophotometer at 240 nm for 1 min at 15-second intervals.

Measurement of GSH, GT, GSSG, and GST.

To quantify the levels of reduced glutathione (GSH) and total glutathione (GT),

the protocol proposed by Rahman, Kode and Biswas [33] was carried out with modifications, using 5,5'-ditiobis 20-nitrobenzoic acid (DTNB) in the homogenate, evidenced by yellow formation. To determine total glutathione (TG) levels, DTNB, nicotinamide-adenine dinucleotide phosphate (NADPH), and glutathione reductase were used in the homogenate. GT and GSH levels were measured at 412 nm (Multiskan GO, Thermo Scientific), and results were expressed as µmol/mg protein. GSSG levels were calculated [34], considering the stoichiometry of the reactions. To evaluate glutathione S-transferase (GST) activity, 1-chloro-2,4-dinitrobenzene (CDNB) and potassium phosphate buffer were used. The absorbance of the samples was measured at 340 nm for 160 seconds, with 5 measurements at 40-second intervals [35].

In silico molecular blind docking

The target protein used in this *in silico* study was human liver mitochondrial catalase (PDB ID: 8SGV), which was downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) database (https://www.rcsb.org/; accessed on 10 January 2025) [36]. Catalase is a tetramer composed of four identical subunits (A,B,C, and D) with over 500 amino acid residues. Each chain is coordinate with iron-heme ring and binds NADPH as a cofactor. We conducted our analysis considering the A subunit isolated. The preparation of the protein for the molecular docking was conducted in the UCSF ChimeraX software (version 1.8) [37]. Water molecules, nonstandard residues and the chains B, C and D were deleted. The 3D structure of melatonin molecule (CID: 896) was downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/; accessed on 10 January 2025) [38] and was prepared in the Avogrado software (version 1.2.0) by adding hydrogens and optimizing the molecule geometry. The next steps were done in the Autodock Tools software (MGLTools-1.5.6). In that stage, any residual water molecule was deleted, polar hydrogens and Kollman charges were attached in the protein. The melatonin structure was forced to let all rotatable bonds rotatable. The grid box was set to 126, 126, and 126 Å along the X-, Y-, and Z-axis with a grid spacing of 0.925 Å in order to recognize the whole catalase subunit. The AutoDocking parameters adopted were: GA runs = 500; population size = 150; maximum number of energy evaluations = 25.000.000; GA crossover mode = two points. The Lamarckian Genetic algorithm was selected to search for the best conformations and the lowest binding energy conformer was chosen for further analysis. The Chimera X and the Biovia Discovery Studio Visualizer (version 24.1.0) were used to analyze and visualize the docking results.

Statistical analysis

Data were processed using an analysis of variance (One-way ANOVA) for one factor and presented as the mean ± standard deviation (SD). Significant results were complemented by Tukey's test. Statistical significance was set at $P < 0.05$ for all analyses. Results were analyzed and generated using GraphPad Prism 9.0 scientific graphing software (GraphPad Software, San Diego, CA).

Results

Mel treatment enhances its intracellular concentration in OC cells.

Based on MTT analysis, we identified the cytotoxic effect of Mel (IC_{50}) which was previously established at 3.4 μM for SKOV-3 cells and 7 μM for CAISMOV-24 cells. Luzindole, an antagonist of MT1/2 Mel receptors, was used to determine whether the Mel's effect is dependent or independent of their membrane receptors. The intracellular concentrations of Mel were measured in the OC cells by the enzyme assay. Mel treatment elevated the levels of intracellular Mel compared to the control group, especially in SKOV-3 cells. After blocking MT1/2 receptors, Mel promoted an increase in its own intracellular levels in both SKOV-3 and CAISMOV-24 cells (1.96-fold increase vs. Control and 2.08-fold increase vs. Control, respectively). More importantly, Mel alone in the presence of its receptors, increased its intracellular levels in SKOV-3 cells (1.75-fold increase vs. Control), thus restoring Mel concentration in OC cells where they are normally depressed (Fig. 1).

Mel reduces the migratory and invasive capacity of SKOV-3 and CAISMOV-24 cells regardless of the MT1/2 receptor activation.

To investigate the effects of Mel and luzindole on the migratory and invasive potential of SKOV-3 and CAISMOV-24 cells, we used transwell inserts in 24-well plates. The invasive potential of SKOV-3 and CAISMOV-24 cells was reduced by Mel alone (2.45- and 3.58-fold decrease vs Control groups, respectively), and after the combination of Mel and luzindole (2.17-fold decrease vs Control group in SKOV-3 cells and 2.38-fold decrease vs Control group in CAISMOV-24 cells; Figs. 2 and 3A, B). The results also showed that Mel alone significantly reduced the migration of both SKOV-3 and CAISMOV-24 cells (2.03- and 2.29-fold decrease vs Control group, respectively; Figs. 2 and 3C, D). When Mel was combined with luzindole, a significant decrease in cell migration was also observed considering both OC cells (1.67-fold decrease vs. Control in SKOV-3 cells and 2.02-fold decrease vs. Control in CAISMOV-24).

Mel differentially regulates the antioxidant enzymes in OC cells.

SOD levels were only reduced by Mel in combination with luzindole in the CAISMOV-24 cells ($p = 0.027$). Regarding Catalase levels, a significant reduction was observed after Mel treatment in both OC cells ($p < 0.05$ for CAISMOV-24 and $p = 0.012$ for SKOV-3). The presence of luzindole restored catalase levels close to that of control groups, thus showing a receptor dependent response; it has been noted that the action of melatonin on antioxidant enzymes are receptor mediated. The changes in glutathione activities following treatments with Mel and luzindole varied differently between the two OC cell lines (Figs. 4 and 5). In SKOV-3 cells, Mel alone significantly decreased the levels of TG ($p < 0.001$) and GSSG ($p < 0.001$) compared with the control group. The combination of Mel and luzindole also showed a decrease in the levels of TG ($p = 0.003$) and GSSG ($p < 0.001$). Conversely, this combination increased the levels of GST compared with control group ($p < 0.001$) and Mel alone ($p = 0.002$). Notably, in CAISMOV-24 cells, Mel alone or combined with luzindole significantly reduced the activities of TG ($p < 0.05$), GST ($p < 0.05$), GSSG ($p < 0.05$), and GSH ($p < 0.05$) compared with the control group. These results demonstrate an opposite effect of Mel favoring pro-oxidative processes in the OC cells.

Mel directly interacts with both the protein portion of catalase and its heme group.

Through in vitro molecular docking assays, the ability of melatonin to interact with catalase was observed. Out of the 500 poses tested, the five with the lowest binding energies are presented in Table 1. Subsequently, experiments were conducted to demonstrate the physical interaction between melatonin and catalase. Melatonin bound to an enzyme pocket in the β barrel domain, very close to the binding site of the heme prosthetic group (Fig. 6A). Melatonin directly interacts with the polypeptide chain of catalase through hydrogen bonds involving residues Arg72, Tyr358, His362, and Arg365. Additionally, alkyl interactions with Val73, Val74, and Val146, as well as π-interactions with His75, are also observed (Fig. 6B). As melatonin was found to be positioned near the porphyrin ring (Fig. 7A), we further docked the interaction between these two molecules. The results show a close distance of approximately 7 Å between melatonin and the heme group, suggesting that the structures are likely oriented in a specific way that facilitates interaction (Fig. 7B). Finally, it can be observed that some amino acids involved in anchoring the porphyrin ring to the protein may also interact with the indolamine (Fig. 8). These potential interactions are of significant value and warrant further investigation.

Table 1
Docking results for interaction between the Melatonin and Catalase (subunit A).

Pose	BE	K_i	vdW + Hbond + desolv	Eletrostatic	Torsional	Unbound
453	-5.99	40.74	-6.99	-0.19	+1.19	-0.49
82	-5.92	45.87	-6.66	-0.45	+1.19	-0.71
214	-5.72	64.28	-6.93	+ 0.02	+1.19	-0.38
146	-5.71	65.11	-6.93	+ 0.03	+1.19	-0.33
475	-5.57	82.37	-6.77	+ 0.01	+1.19	-0.37

BE: Binding energy; Ki: Estimated Inhibition Constant (micromole. L^{-1}); vdW: van der Waals interactions; desolv: desolvation energy. Energy parameters are expressed as kcal.mol^{-1}.

Discussion

Herein we report that exogenously applied melatonin to the incubation medium increases the uptake of melatonin to improve its intracellular levels accompanied by attenuation of the invasive and migratory capacity of OC cells in a receptor-independent manner. Melatonin has been shown to be taken up by cells [39]. We also bring new information regarding the interference of Mel in the enzymatic antioxidant system of OC cells which may negatively impact their survival and growth (Fig. 9). Firstly, we observed an increased concentration of Mel in both OC cell lines, mainly in SKOV-3 cells following Mel treatment, in the presence of Luz or not, and in CAISMOV-24 cells only after the combination of Mel and Luz. We

previously documented an increase in the intracellular concentrations of Mel in SKOV-3 cells after treatment with 3.2 mM of Mel [40]. A greater Mel level may contribute to the antiangiogenic process, pro-oxidative, and pro-apoptotic pathways and may promote modifications in the metabolic profile of tumor cells [41]. The elevation in intracellular levels of Mel appear to be receptor-independent since the combination of Mel with luzindole also promoted an increase in the availability of the indolamine. Treatment with Mel can disinhibit the enzymatic activity of the pyruvate dehydrogenase complex (PDC), either directly [42] or by first reducing hypoxia-inducible factor 1-alpha (HIF-1α), a factor highly expressed in hypoxic conditions of the tumor microenvironment, which stimulates the pyruvate dehydrogenase kinase (PDK) enzyme. After PDC disinhibition, Mel can be synthesized in mitochondria, and oxidative phosphorylation is reestablished [40, 43].

The fact that Mel promotes a reduction in the invasive and migratory capacity of OC cells is well known [44–46]. Recently, our group showed that MT1 knockdown in SKOV-3 cells treated with 3.2- or 4-mM Mel presented a remarkable reduction in cell migration and invasion [46]; we obtained similar results using luzindole, a non-selective antagonist of both MT1 and MT2 receptors, in two different OC cell lines via transwell inserts. Despite the use of concentrations ranging from 3.4 and 7 μM of Mel to SKOV-3 and CAISMOV-24 cells, respectively, our results clearly demonstrated a reduction in the invasive and migratory capacity of OC cells, regardless of the activation of Mel receptors. The precise mechanisms by which Mel regulates OC cell migration and invasion are not yet fully understood. Previous studies have shown that Mel exerts anti-migratory effects via the MAPK and PI3K pathways in OC cells [44, 46]. In other cancer types, such as colon and breast cancer, Mel attenuated cell invasion, migration, and survival by targeting PI3K/AKT and NF-κB signaling pathway [47] or by downregulating the p38 MAPK pathway [48]. In addition to its passive diffusion, Mel can alternatively enter tumor cells using other membrane transporters such as PEPT1/2 and GLUT1 [46, 49], therefore it may act in an MT1/2 receptor-independent manner to exert the anti-tumoral effects on the migratory and invasive potential of OC cells.

Defects in mitochondrial metabolism can increase the production of ROS and RNS, leading to OS. To combat the excessive and harmful production of these radical and non-radical species, cells are equipped with an antioxidant defense system that includes enzymes such as SOD, CAT, and glutathione derivatives [50]. An excessive and permanent generation of OS is related to genetic/epigenetic events that could facilitate the tumorigenic process; however, increasing the generation of ROS and RNS in tumor cells is paradoxically used as a strategy to induce their death [51, 52]. A potential actor in this approach is the use of Mel, known for its antiproliferative effects and for interfering with angiogenesis and metastasis events; Mel acts by modulating the oxidant/antioxidant state of cells [52–56]. Moreover, the effects of Mel on tumor cells seem to be opposite of those occurring in normal healthy cells, that is, Mel acts as a pro-oxidant agent in tumor cells, thus reducing their antioxidant defenses [57, 58].

Our results reinforce the apparent pro-oxidant properties of Mel in OC cells, especially in CAISMOV-24 cells. We are the first to demonstrate a decrease in SOD activity when Mel was combined with luzindole, i.e., a receptor-independent response, and a significant reduction in CAT levels after Mel treatment in both OC cells, suggesting that Mel acts directly as a modulator of these two antioxidant enzymes. Mel

treatments have also been associated with lower enzymatic activities of SOD and CAT accompanied by an increased level of ROS observed in human colorectal cancer cells and in the hepatocellular carcinoma cells [59, 60], resulting in apoptosis due to an excessive oxidative damage.

In healthy tissues, Mel generally upregulates antioxidant enzymes, including the glutathione system that plays a central role against OS [56, 61]. Here, we observed a marked decrease in total glutathione (GT) levels in both OC cell lines following treatment with Mel alone. When Mel receptors were blocked by luzindole, only SKOV-3 cells showed a significant reduction in GT levels, while CAISMOV-24 cells showed only a trend. The reduced form of glutathione (GSH) is one of the most important machineries on the front line of the antioxidant defense system. GSH maintains a redox state in subcellular compartments such as mitochondria and cytosol, and its increased levels may be involved in the chemoresistance of cancer cells [62]. In this study, GSH content was only reduced with CAISMOV-24 cells treated with Mel alone. It is also reported that Mel can exert a depletion on GSH levels in HepG2 cells [52, 63], and in human myeloid leukemia cell line (U937) [60, 64]. Under OS conditions, GSH can be directly converted to oxidized glutathione (GSSG) in the presence of ROS [52]. We observed a notable reduction in GSSG levels in both OC cells. Particularly, for SKOV-3 cells, GSH levels were like that of the control group, while GSSG was significantly decreased by both treatments. Based on this GSH:GSSG ratio that favors GSH, we can hypothesize a more pronounced resistance of SKOV-3 cells to OS, which leads to the maintenance of GSH homeostasis, differing from what was observed with CAISMOV-24 cells.

Cancer cells can also gain resistance through overexpression of enzymes that can increase detoxification capacity and avoid the cytotoxic action of antitumor drugs [65]. More specifically, overexpression of glutathione S-transferases (GST) and efflux pumps in tumor cells can reduce the reactivity of various anticancer drugs, such as cyclophosphamide [66], cisplatin [67], and others [65]. In this study, OC cell lines showed opposite results regarding GST. While in CAISMOV-24 cells both treatments decreased GST activities, in SKOV-3 cells the combination of Mel with luzindole increased its activity. As reviewed in detail by Sau et al. [65], the increase in GST levels occurs through transcriptional activation mediated by the nuclear factor erythroid 2 p45-related factor 2 (Nrf2). However, further studies are needed to precisely determine the role of Mel and its receptors in the molecular pathways that regulate the activity and expression of genes in the glutathione system. Overexpression of GST and high levels of GSH are linked to the development and expression of chemoresistance [68]. In this context, our treatment with Mel alone at 7 μM successfully promotes a decrease in GST and GSH levels in CAISMOV-24, a recently established human low-grade serous ovarian carcinoma cell line [69]. SKOV-3 cells, a non-serous ovarian cancer cell line, in turn, remained unchanged for these parameters after treatment of 3.4 μM of Mel alone, thus suggesting further evaluations with higher concentrations. This sounds plausible since it has been shown that high levels of Mel are required to induce ROS production in tumor cells [27]. In our previous study using the same OC cell lines and melatonin concentrations, we observed a reduction in Warburg-type metabolism and potentially glutaminolysis, which further attenuated oncogenic targets associated with OC progression and invasion [70].

As stated below, melatonin's capacity to modulate antioxidant enzymes activity is well documented. However, it remains unclear whether melatonin interacts directly with specific enzymes, and the precise nature of such interactions has not been fully explored. To bridge this gap, and given that catalase activity was reduced in the two OC cell lines, we employed in silico molecular docking to investigate the interaction between melatonin and catalase. Catalase's catalytic mechanism occurs in two stages. Initially, the heme Fe^{3+} reduces a hydrogen peroxide molecule to water, forming a covalent oxyferryl species, along with a porphyrin π-cation radical. This radical then oxidizes another hydrogen peroxide molecule into molecular oxygen, while the ferryl oxygen species is released as water. Several amino acid residues, including tyrosine, play crucial roles in facilitating these reactions at the active site [71].

The docking results revealed that melatonin binds most effectively on a structural pocket between β-sheet and wrapping domain, near the heme-binding cavity of catalase. Melatonin binding was stabilized with hydrogens and hydrophobic interactions, with a binding energy of -5.99 $kcal.mol^{-1}$. The amino acid residues Arg72, Tyr, 358, His362, and Arg365 play major role in the binding of melatonin on catalase subunit. Melatonin forms four conventional hydrogen bonds with those amino acids, along with additional potential interactions. Notably, the tyrosine residue at position 358 interacts closely with the iron in the porphyrin ring, and melatonin also engages with this residue. Given the critical role of the heme group in catalase's catalytic activity, this interaction with Tyr358 may provide valuable insight into the mechanism by which melatonin reduces the enzyme's activity. Additionally, melatonin's interaction with other amino acid residues could alter the microenvironment of the catalase active site, further contributing to the observed effect in enzyme activity. The binding of farnesiferol C, a sesquiterpene coumarin, to bovine liver catalase yielded similar findings, affecting both the enzyme's affinity and catalytic activity [72]. The discovery of specific, non-toxic catalase inhibitors presents significant potential for use in cancer co-therapy. For instance, a Zn(II) complex tested against human colon cancer cells demonstrated cytotoxic effects by inhibiting catalase through mixed-type inhibition kinetics. Interestingly, the binding site of the complex also involved hydrogen bonding and exhibited a free energy of -7.13 kcal/mol [73], similar to the binding characteristics observed for melatonin.

An intriguing and unexpected finding in our study was the close proximity of melatonin to the heme group of catalase. To explore this further, we evaluated the potential physical interaction between melatonin and the porphyrin ring. The binding free energy between melatonin and the heme group was calculated to be -4.68 kcal/mol, suggesting various interaction types. More importantly, several amino acid residues critical for maintaining the stability of the heme group – such as Val73, Val74, Arg72, His75, and Val146 – were found to interact with melatonin. These interactions may contribute to melatonin's inhibition of catalase activity, a possibility that warrants further investigation.

Conclusion

Collectively, Mel treatment attenuates the migratory and invasive capacity of OC cells in a receptor independent manner while stimulating its intracellular concentration. Moreover, the antioxidant enzymatic defenses were dampened by Mel, especially in the CAISMOV-24 cells. The blockage of MT1/2

receptors by the antagonist luzindole followed by Mel administration showed a tendency to soften the results. Indeed, pre-incubation of SKOV-3 cells with 10 μM luzindole revealed an increase in cell viability [44]. Based on our results, we believe that the function of Mel in SKOV3 and CAISMOV-24 is partially mediated by MT1 and MT2 receptors. Also, our data provide valuable insights into the regulatory role of Mel in modulating antioxidant status in OC cells. Based on the molecular docking study, future investigations using molecular dynamics simulations and enzyme kinetics assays will be valuable and necessary to confirm the predicted interactions between catalase and melatonin as pivotal to enzyme modulation.

Declarations

Conflicts of Interest:

The authors declare no competing interest.

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Author Contributions:

Conceptualization, HS, FRS and LGAC; Data curation, HS; Formal analysis: RCC, FG, FRS, VAS, GSAF, MCS, DAPCZ and LGAC; Investigation, HS, FRS and DAPCZ; Methodology, HS, RCC, FG and DAPCZ; Visualization, RR; Writing – original draft, HS and LGAC; Writing – review & editing, RR. The authors read and approved the final version of this manuscript.

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Figures

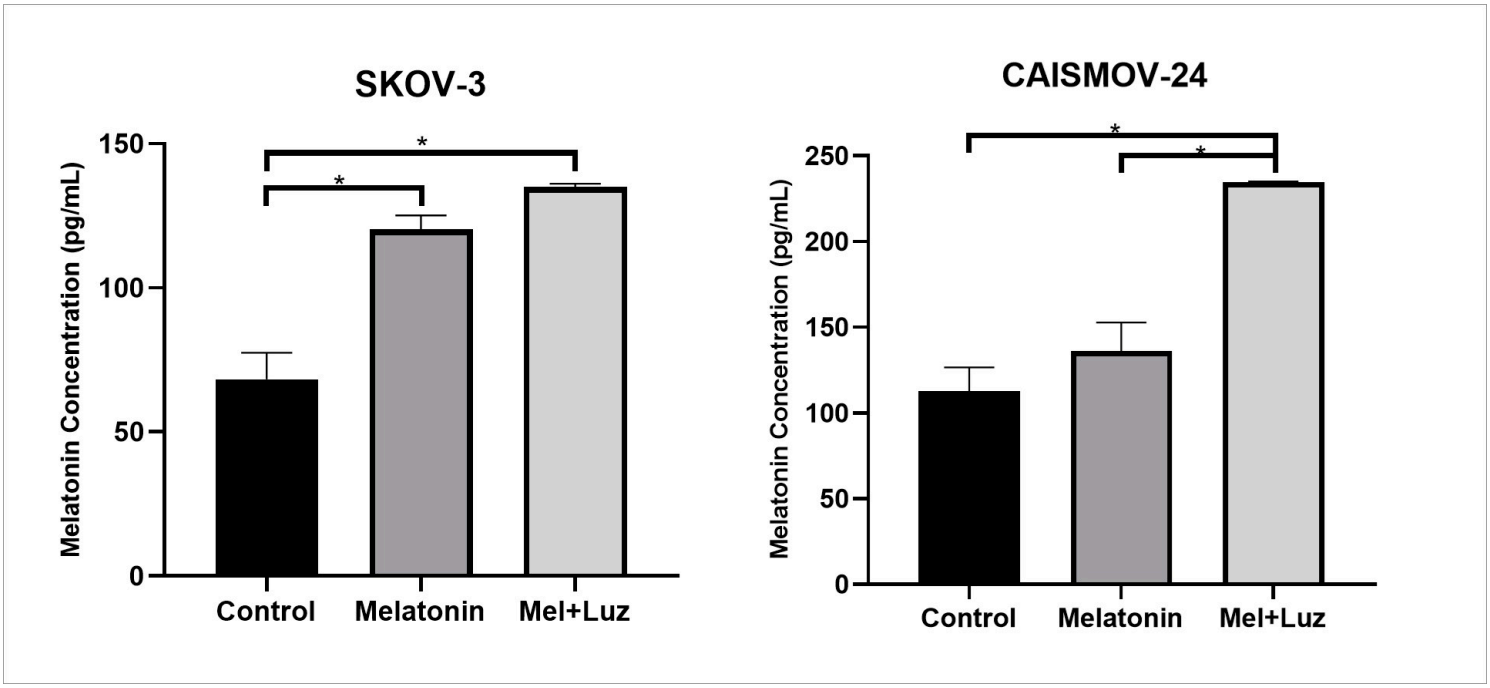

Figure 1

Intracellular Mel concentration after treatment with Mel and Luz in the SKOV-3 and CAISMOV-24 cell lines. Data were expressed as mean ± SEM of triplets. * P<0.05.

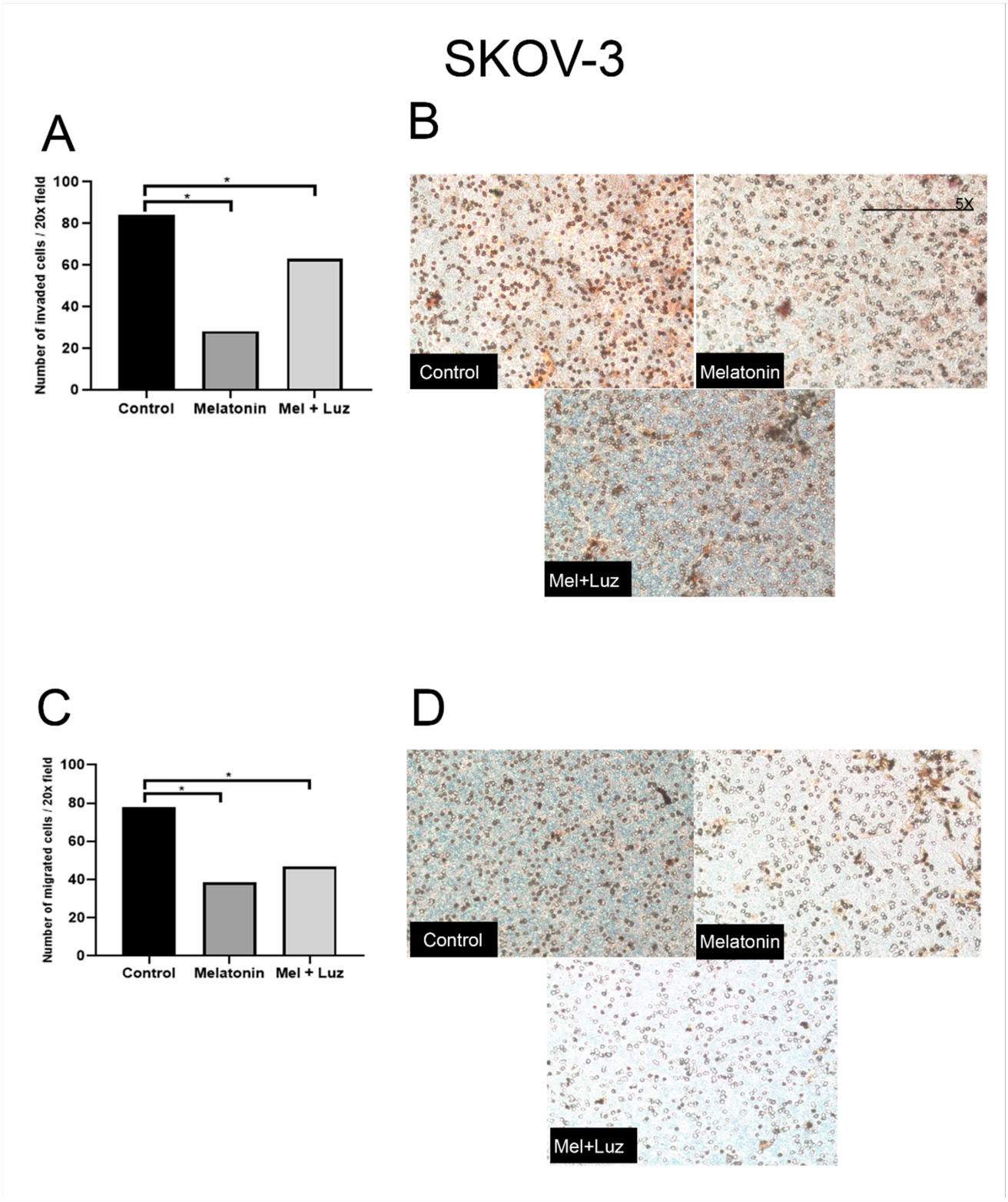

Figure 2

Effects of Mel and Luz on the invasion and migration of SKOV-3 cells. A) Effect of Mel and Luz on the invasive capacity of SKOV-3 cells. B) Images of invaded cells after treatments. C) Effect of Mel and Luz on the migratory potential of SKOV-3 cells. D) Images of migrated cells after treatments. Mel: melatonin; Luz: luzindole. Data were expressed as mean ± SEM of the triplets. *P<0.05.

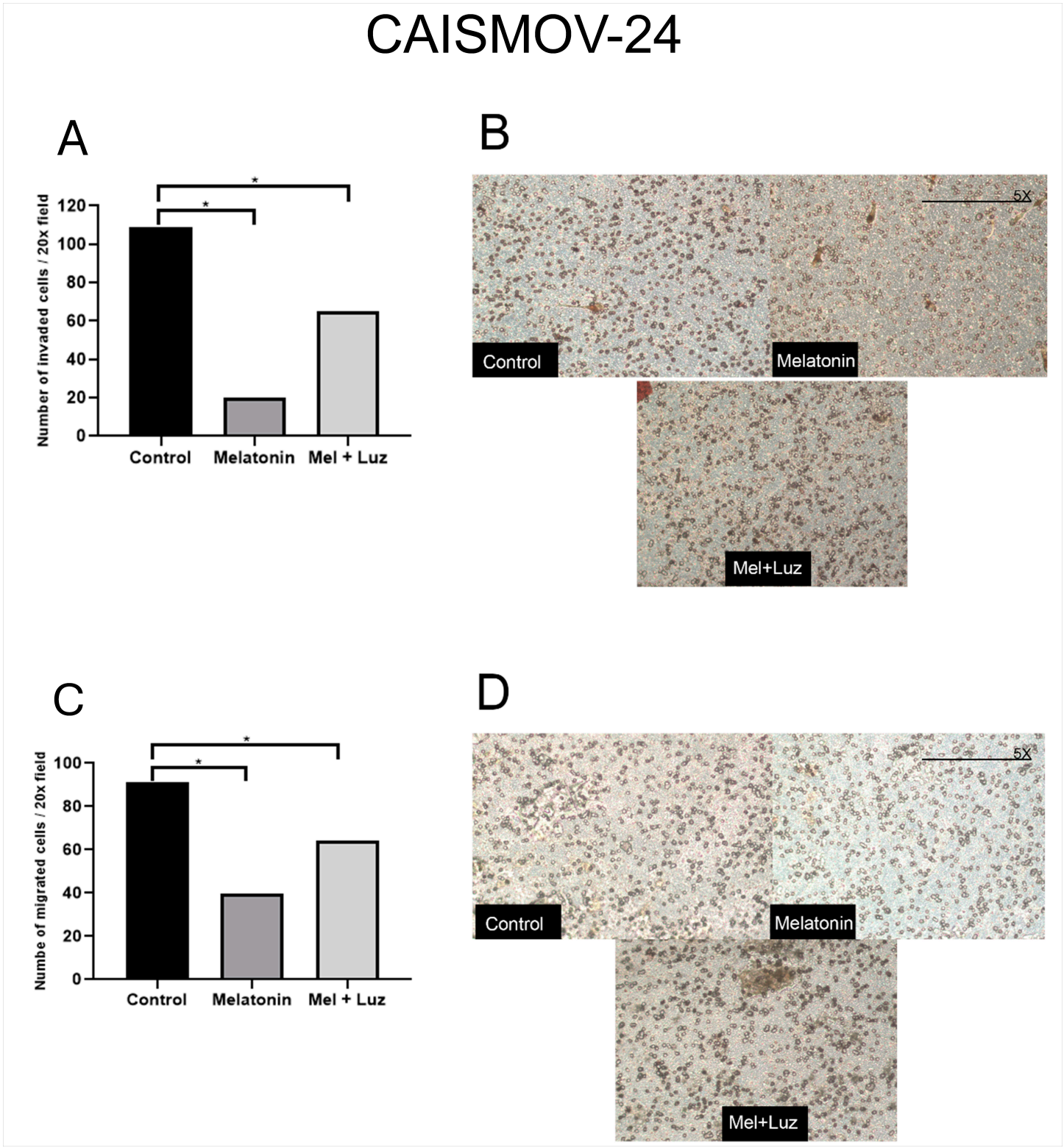

Figure 3

Effects of Mel and Luzon the invasion and migration of CAISMOV-24 cells. A) Effect of Mel and Luz on the invasive capacity of CAISMOV-24 cells. B) Images of invaded cells after treatments. C) Effect of Mel and Luz on the migratory potential of CAISMOV-24 cells. D) Images of migrated cells after treatments. Mel: melatonin; Luz: luzindole. Data were expressed as mean ± SEM of the triplets. *P<0.05.

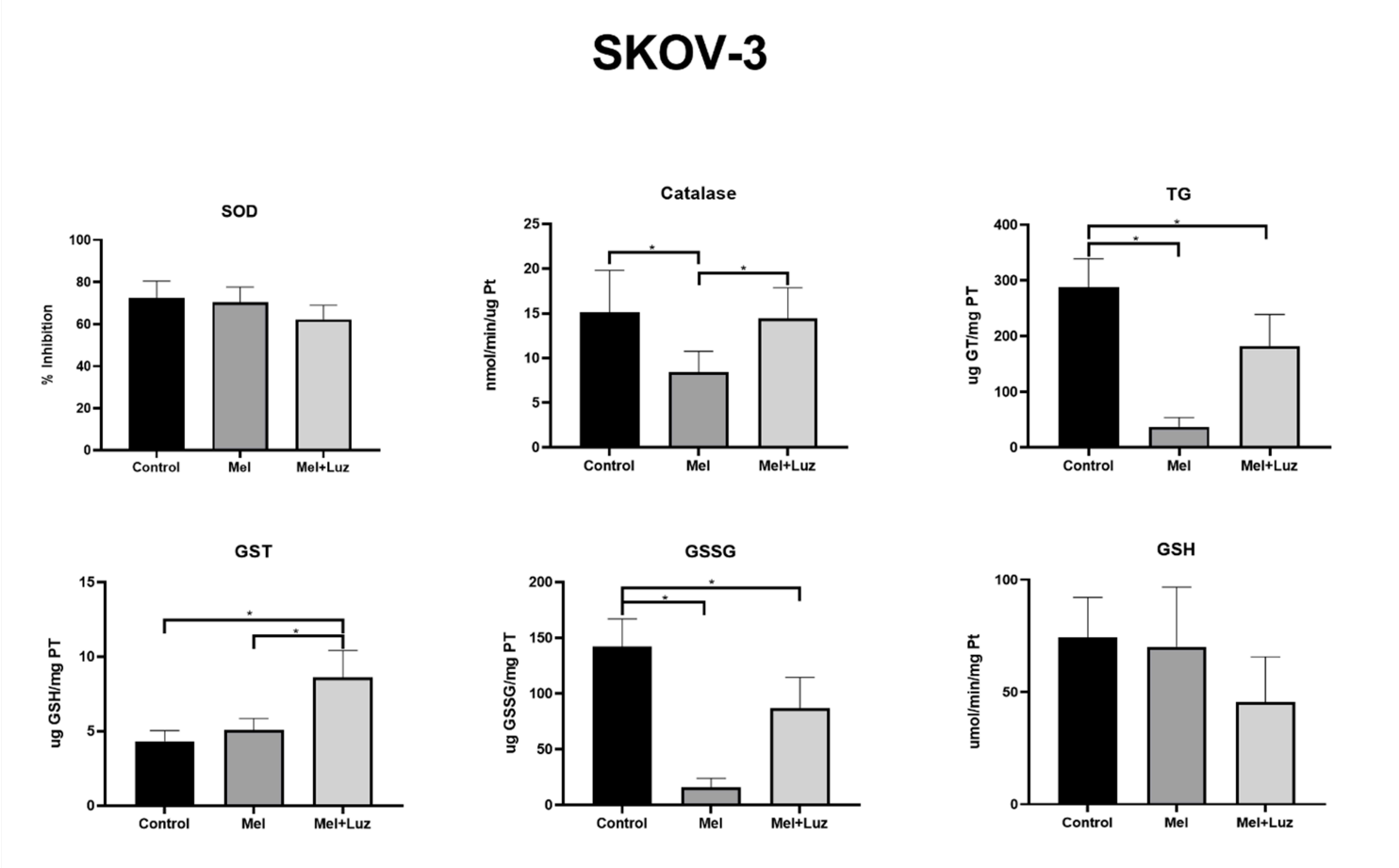

Figure 4

Enzymatic activities of SOD, Catalase, GT, GST, GSSG and GSH in SKOV-3 cells in response to Mel and luzindole after 24h of treatment exposure. Data are expressed as the mean ± SD. *P<0.05. All samples were assayed in triplicate and in the same run. One-way ANOVA complemented by Tukey's test. SOD: superoxide dismutase, TG: Total Glutathione, GST: Glutathione-S-transferase, GSSG: Oxidized glutathione, GSH: Reduced glutathione.

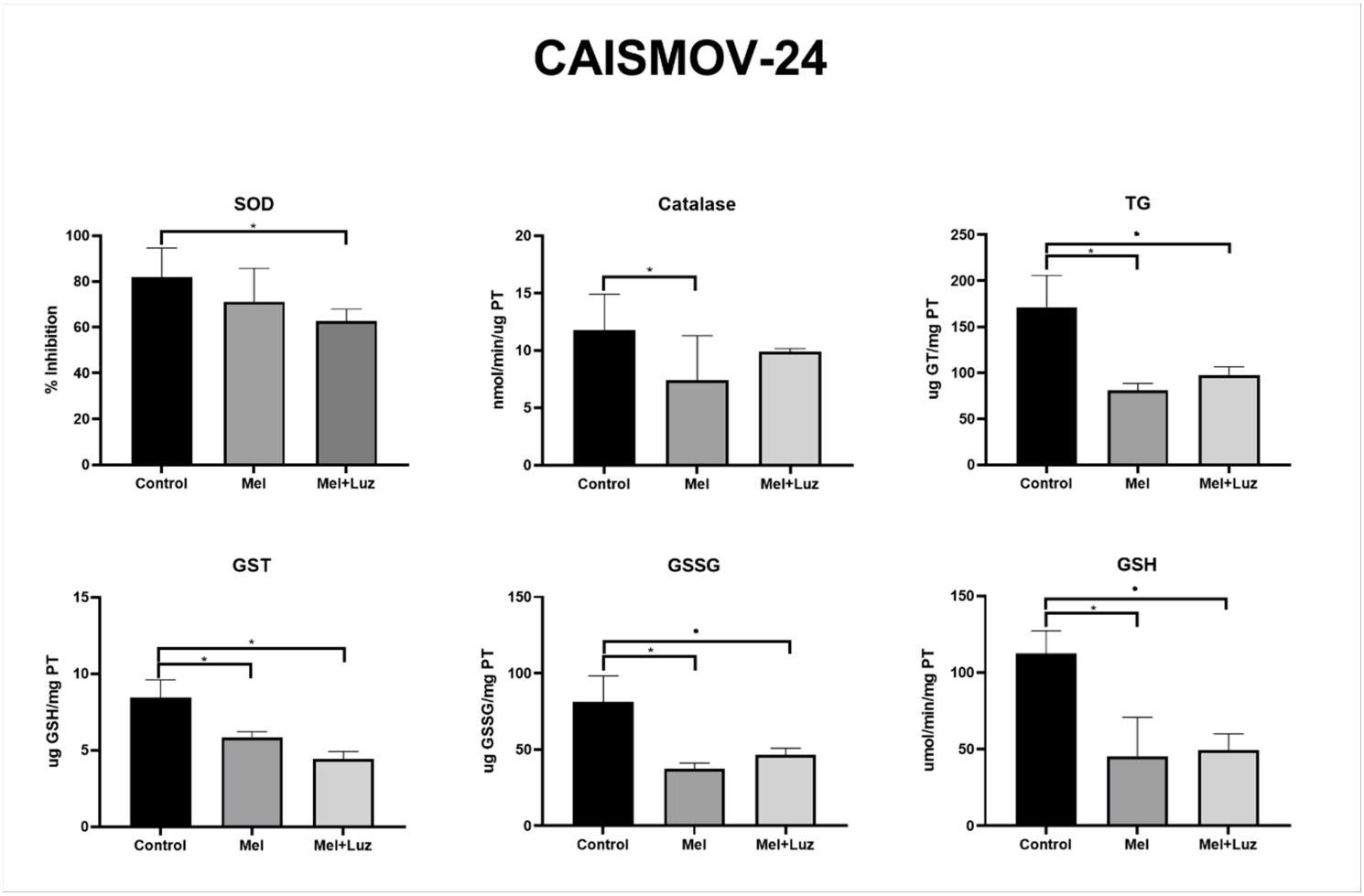

Figure 5

Enzymatic activities of SOD, Catalase, GT, GST, GSSG and GSH in CAISMOV-24 cells in response to Mel and luzindole after 24h of treatment exposure. Data are expressed as the mean ± SD. *P<0.05. All samples were assayed in triplicate and in the same run. One-way ANOVA complemented by Tukey's test. SOD: superoxide dismutase, TG: Total Glutathione, GST: Glutathione-S-transferase, GSSG: Oxidized glutathione, GSH: Reduced glutathione.

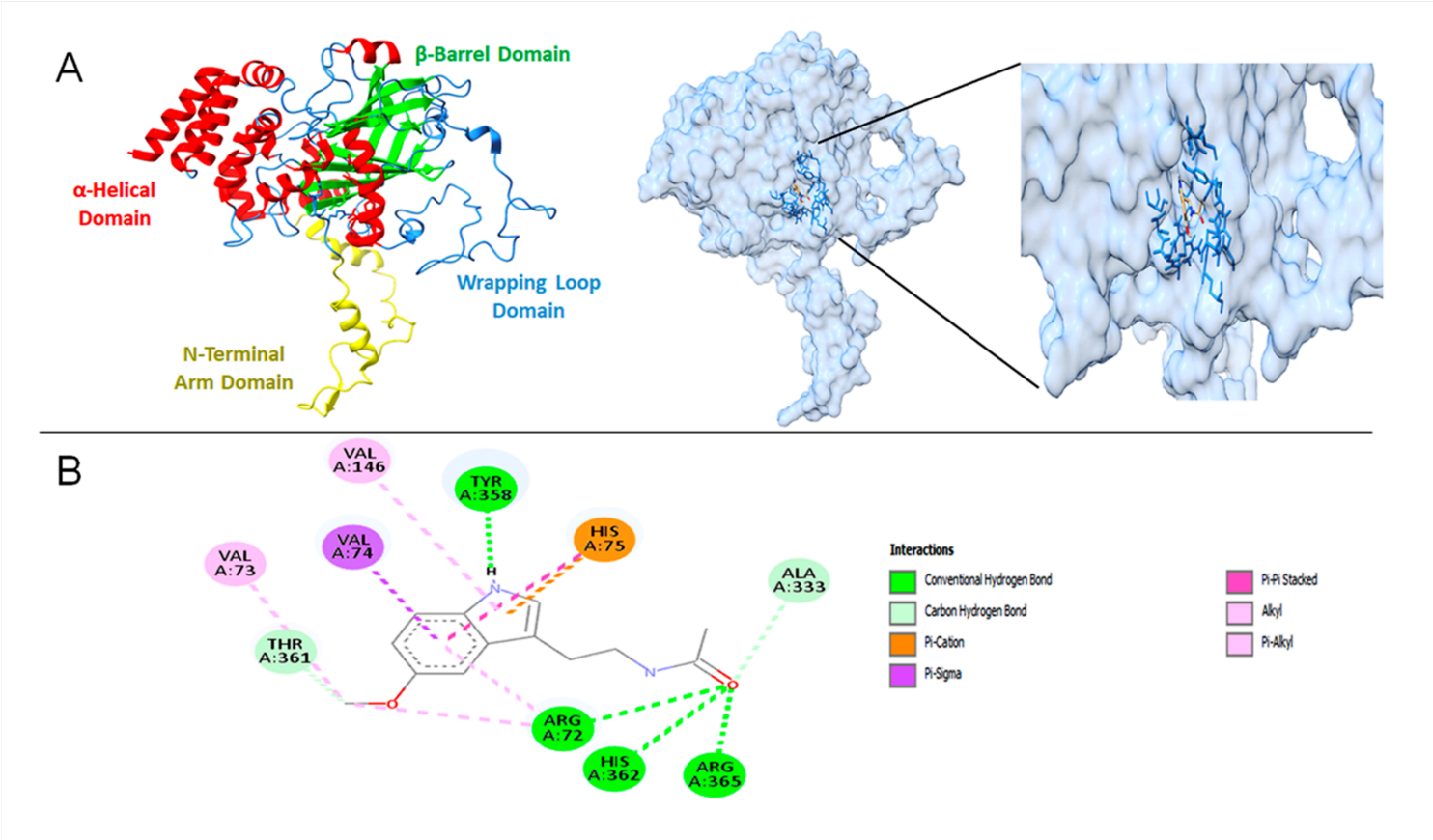

Figure 6

Molecular docking results showing the interaction between melatonin and catalase. A) Structure of a catalase subunit evidencing its domains and melatonin localization. B) Specific amino acids residues involved in melatonin binding.

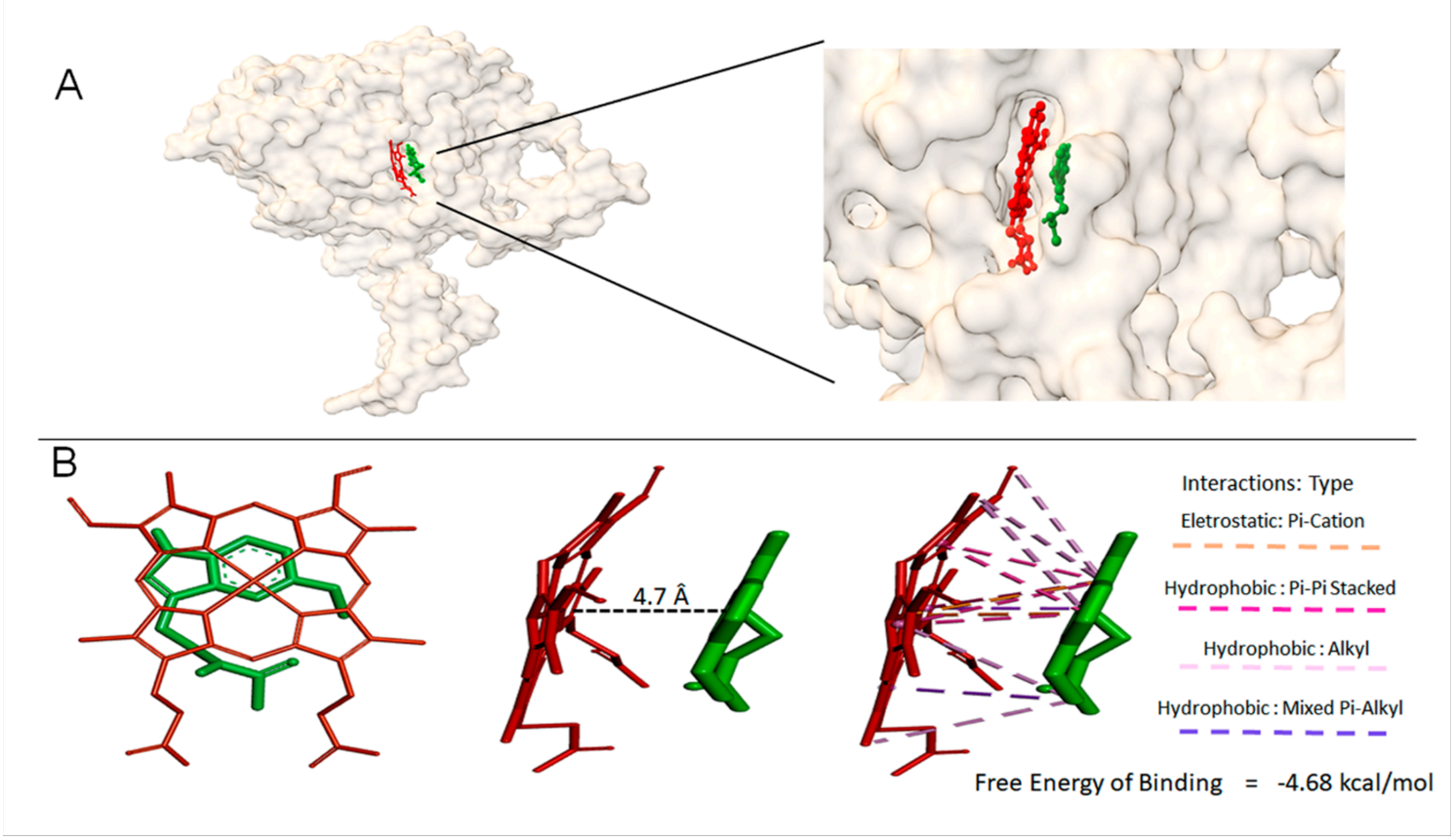

Figure 7

Docking view illustrating the close association between melatonin (green) and the heme group (red). A) Melatonin binds in the same pocket as the heme group. B) Theoretical interaction between melatonin and the heme group.

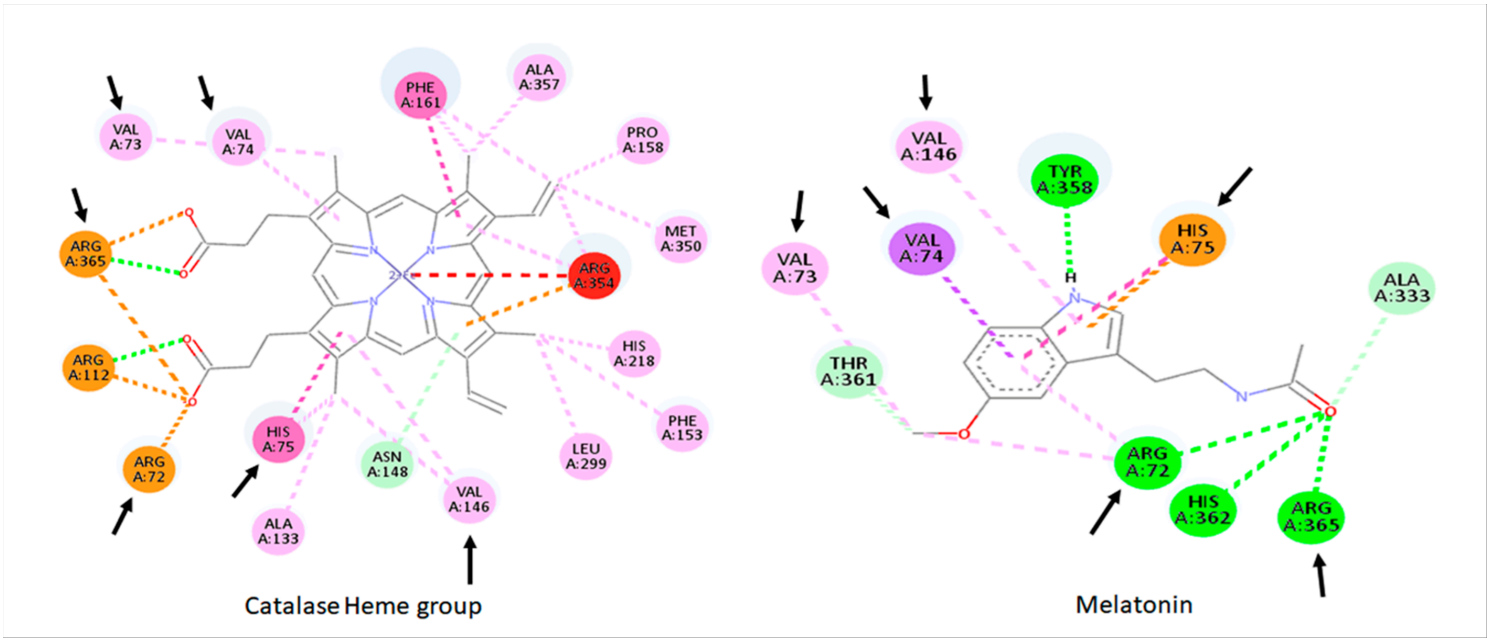

Figure 8

Common amino acid residues (indicated by arrows) that form heme-binding site and may also contribute to melatonin binding.

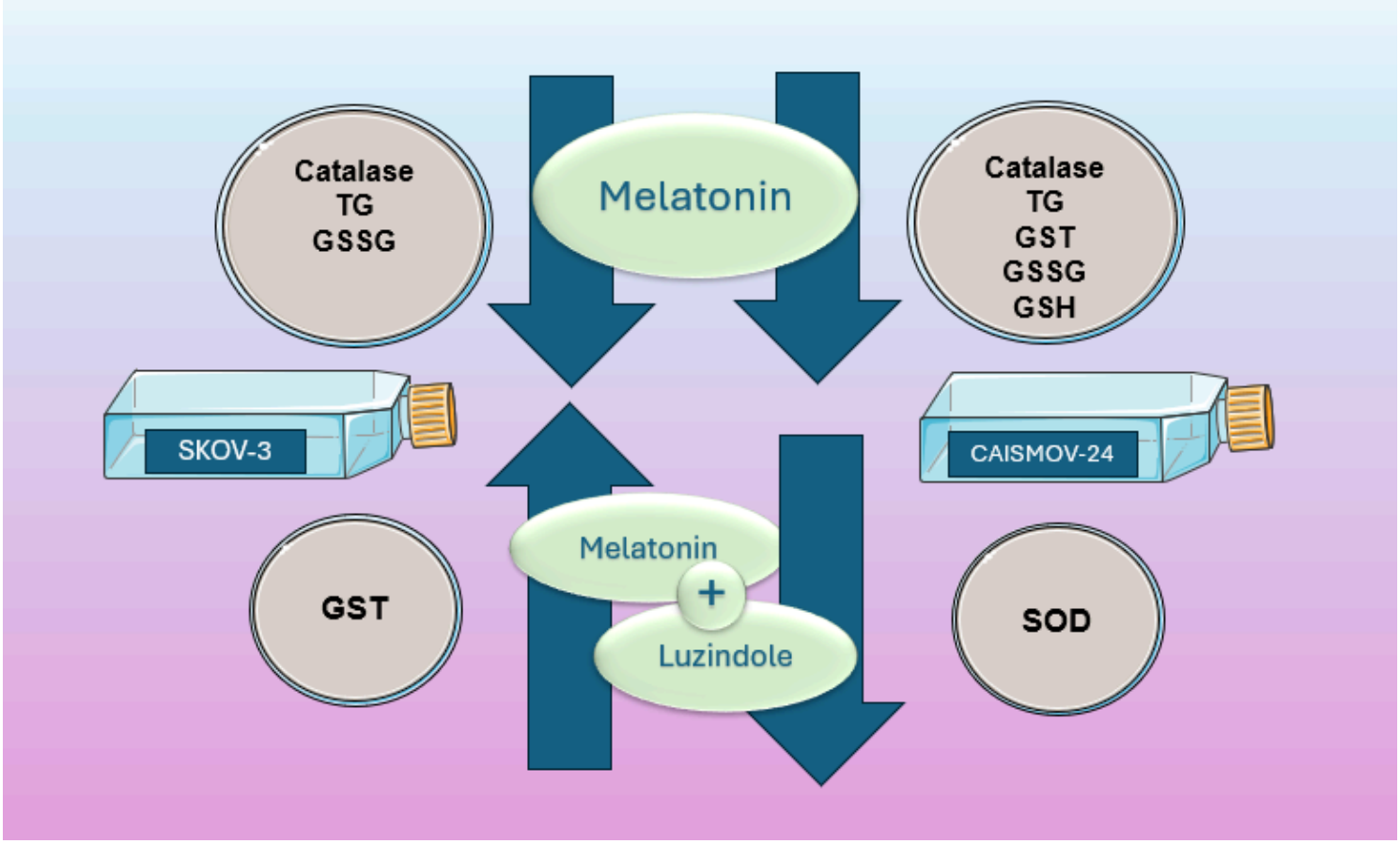

Figure 9

Schematic representation of the effects of Mel against agents related to oxidative stress in SKOV-3 and CAISMOV-24 OC. SOD: superoxide dismutase, GT: Total Glutathione, GST: Glutathione-S-transferase, GSSG: Oxidized glutathione, GSH: Reduced glutathione.