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Article - Human and Animal Health

Curcumin and Vinblastine Disturb Ectonucleotides Enzymes Activity and Promote ROS Production in Human Cutaneous Melanoma Cells

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HIGHLIGHTS

- Melanoma is a cancer with poor prognosis, and metastasis.
- Curcumin is a compound with many beneficial effects regarding prevention and adjuvant treatment of cancer
- Vinblastine and curcumin greatly promoted production of ROS in melanoma cells
- Curcumin disrupted adenosine formation cascade, reducing imunosupression at tumoral site.

Abstract: Cancer is the leading cause of death. Melanoma skin cancer originates in melanocytes and represents 80% of the deaths associated with skin cancer. Vinblastine (VIN) is a chemotherapeutic agent used in the treatment of cancer through disrupting mitotic spindle and tumor development. Curcumin (CUR), a compound extracted from the rhizomes of the Curcuma longa plant, has beneficial effects in preventing the development and progression of cancer while modulating the immune response and oxidative stress. The expression of purinergic receptors, ecto-enzymes, and adenosine can modulate the inflammatory responses in cancer. The activity of enzymes, the markers of cell damage in oxidative stress, the generation of reactive oxygen species (ROS), and the activities of ecto-enzymes in the melanoma cell line were investigated. The human melanoma cell line was treated with curcumin (40 µM), vinblastine (VIN) (20 nM), or a combination of both for 24h. Oxidative stress enzymes and byproducts were measured and compared against the activity of ecto-enzymes. There was a marked increase in ROS production in all groups, but an increase in protein carbonylation was only detected in the VIN group. CUR had an inhibitory effect on extracellular ADP

hydrolysis, as evidenced by a significant decrease in ADP substrate removal. VIN possibly increased adenosine formation, as demonstrated by an increase in ADP substrate removal. VIN (alone or in combination with CUR) reduced the activity of ADA, thus increasing the concentration of adenosine in the tumor microenvironment. CUR increased ROS generation in melanoma cells and disrupted the purinergic signaling cascade. Therefore, it may be a promising adjuvant therapy for melanoma, a cancer with a high incidence and lethality.

Keywords: melanoma; curcumin; skin; enzymes; adjuvant; treatment.

INTRODUCTION

Cancer is a leading cause of death, responsible for more than 10 million deaths in 2019 [1]. Cancer develops through a series of successive mutations in cellular genes wherein cancer cells form [2]. Melanoma skin cancer originates in melanocytes and represents 80% of deaths associated with skin cancer [3]. Melanoma results from a multifactorial process that involves genetic, phenotypic, and environmental predisposition [4]. Vinblastine (VIN) is a natural compound from Catharanthus roseus, known as Madagascar periwinkle, used in the treatment of melanoma and other tumors, targeting cellular tubulin [5] and impairing mitotic spindle and tumor development.

Inflammation is an important risk factor for cancer development [6]. Since oxidative stress and inflammation are linked, when cellular antioxidant systems fail to maintain cell homeostasis, it may cause damage to cell components, promote inflammation, and alter many pathways, such as purinergic signaling [7].

The expression of purinergic receptors, ecto-enzymes, and adenosine can modulate the inflammatory response in cancer. When ATP is present in the extracellular space of the tumor microenvironment, it is rapidly hydrolyzed by the enzyme CD39/NTPDase. AMP is hydrolyzed by ecto-5nucleotidase/CD73 to adenosine. Adenosine is mainly recognized as an immunosuppressive nucleotide and is targeted for immune treatments [8]. Finally, adenosine is converted to inosine by adenosine deaminase (ADA) [9]. Adenosine maintains an immunosuppressive environment and promotes further cell damage through reactive oxygen species (ROS) generated by inflammatory and cancerous cells.

ROS are highly reactive molecules that are maintained at low levels by a balance between their production and removal. ROS are involved in many physiological and pathological pathways, promoting apoptosis, cancer initiation, and cellular protein damage [10]. In this context, studies have analyzed the effects of natural substances in tumor cells and their potential as an adjuvant cancer therapy in the future.

Curcumin, a compound extracted from the rhizomes of the Curcuma longa plant, has antineoplastic effects, inhibiting tumor initiation [11] and tumor promotion [12] by acting on a wide variety of genes, growth factors, and enzymes that regulate cell proliferation and apoptosis [13]. Furthermore, curcumin has antioxidant, anti-inflammatory [14], neuroprotective [15], anticancer, and antimicrobial capabilities [16], among others, such as the modulation of ecto-enzymes [17]. It is unknown, however, whether curcumin, alone or in combination with chemotherapeutic compounds, has any effect on melanoma cell lines, such as SK-MEL-28 cells, regarding ecto-enzymes and oxidative stress.

Considering the impact of oxidative stress and purinergic signaling on inflammatory conditions such as melanoma, the present study aimed to investigate the activity of enzymes and the markers of cell damage in oxidative stress, ROS generation, and the activity of ecto-enzymes in melanoma cell lines treated with curcumin and/or vinblastine.

MATERIAL AND METHODS

Cell line and cell culture

The human melanoma cell line (SK-MEL-28-ATCC® HTB-72™) was maintained in Dulbecco's Modified Eagle Medium with penicillin (100 IU/mL), streptomycin (100 µg/mL), and 10% fetal bovine serum (tested for cell culture) at 37℃ in a 5% CO2 atmosphere with 95% humidity. The culture medium was renewed two to three times per week.

Preparation of the CUR solution

A solution of 100 mM curcumin (CUR) (Sigma-Aldrich), diluted in dimethylsulfoxide (DMSO), was used at different concentrations: 20, 30, 40, 50, 60, 70, 80, 90, and 100µM. The maximum concentration of DMSO was less than 0.1%.

Cell viability

Cell viability was evaluated using the MTT assay [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide salt] [18], employing CUR from 20 to 100 µM for 24h. Sodium hypochlorite (0.2% w/v) was designated as resulting in 100% death (positive control). The half-maximal inhibitory concentration (IC50) of CUR was obtained through nonlinear regression and was used in subsequent treatments. To compare the effect of CUR to a chemotherapeutic drug used in cancer treatment, we used VIN at 20 nM [19] and a combination of both CUR and VIN. Unless otherwise specified, all cells were treated with CUR, VIN, or both for 24 h.

E-NTPDase, E-5'-nucleotidase, and E-ADA assays

Twenty microliters of cell suspension (0.8–1.0 mg/mL of protein in 0.9%NaCl) was added to the reaction mixture of E-NTPDase or E-5'-nucleotidase and pre-incubated for 10 min at 37 ℃ in a final volume of 200 µL. The activities of E-NTPDase and E-5'-nucleotidase were determined using previously described methods [20,21]. Protein concentrations were measured according to the Bradford method [22]. Enzyme-specific activities are reported as nmol Pi released/min/mg of protein. ADA activity was quantified spectrophotometrically [23]. The amount of ammonia produced was measured by the absorption at 620 nm, and the results are expressed in units/mg of protein.

Oxidative stress assays

Catalase (CAT) and superoxide dismutase (SOD) activity

CAT and SOD activities were determined in lysed cells. CAT activity was quantified (240 nm) by the decomposition of H2O2 (3.4% v/v) [24]. SOD activity was measured (480 nm) by the inhibition of auto-oxidation of epinephrine at alkaline pH [25]. Each assay used 10–40 µg of protein,and the results are expressed in ηmoles/mg of protein relative to the untreated control.

Levels of reactive oxygen species (ROS) measured by flow cytometry

The level of 2-7-dichlorofluorescein (DCFH) relies on the deacetylation of DCFH-DA and the oxidation to DCFHby ROS [26]. SK-MEL-28 cells (10 µL) were added to a medium containing buffer [Tris–HCl, 10 mM Tris-HCl (pH 7.4) and DCFH-DA (1 mM)]. Cells were then maintained in a dark room for 1 h, and flow cytometry was performed (excitation at 488 nm, emission at 525 nm, and slit width set to 1.5 nm).

Thiobarbituric acid reactive substance (TBARS) level

Malondialdehyde (MDA) was used as a lipid peroxidation marker. SK-MEL-28 cells (200 µL) were maintained at 95 ℃ for 1 h in acid medium with 8.1% sodium dodecyl sulfate, 0.5 mL acetic acid buffer (500 mM, pH 3.4), and 0.6% TBA. TBARS levels (532 nm) were compared to a standard curve using MDA [27] and expressed as nmol of MDA per mg of protein.

Protein carbonylation level

Carbonylation of proteins was estimated by the formation of hydrazone products [28] using 2,4-dinitrophenyl hydrazine (DNPH) and was expressed in nmol of carbonyl per mg of protein.

Statistical analysis

Data were analyzed using GraphPad Prism (version 6.01) with one-way ANOVA, followed by Tukey's post hoc test. IC50 was calculated using nonlinear regression. Differences were considered significant at $p \leq 0.05$. Data from three independent experiments are shown as the mean values ± SD.

RESULTS

CUR decreases the viability of SK-MEL-28 cells

We evaluated the antitumor activity of CUR (20, 30, 40, 50, 60, 70, 80, 90, and 100 µM) in a human cutaneous melanoma cell line (SK-MEL-28) for 24 h. After the MTT assay, we obtained a cytotoxic concentration-response graph and identified that all tested CUR concentrations (Figure 1) decreased tumor cell viability (one-way ANOVA: $F_{9,23} = 121.4$, $p<0.0001$). However, CUR decreased cell viability to 50% only at the concentration of 40 µM (nonlinear regression), which was therefore defined as a 50% inhibitory

concentration (IC50) in 24 h. A Tukey's post hoc test demonstrated that the cell viability at 40 µM was statistically different than those at 20 µM , 50 µM, 60 µM , or higher concentrations tested.

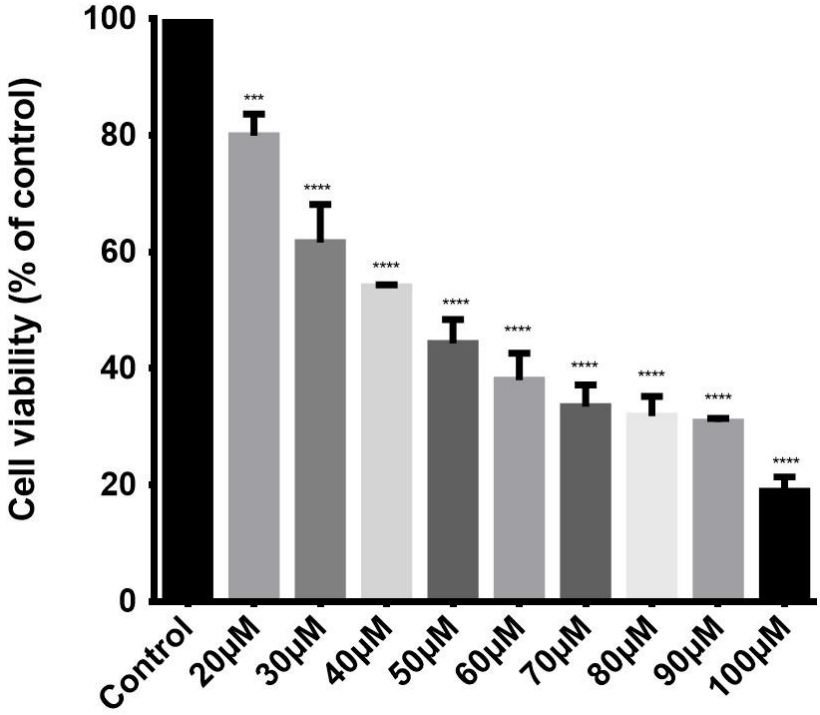

Figure 1. MTT assay demonstrated decreased viability of SK-MEL-28 cells after exposure to increasing concentrations of CUR (20, 30, 40, 50, 60, 70,0 80 and 100 µM) for 24 h. 40 µM concentration was statistically different of 20 µM (p=0.0001), 50 µM (p=0.035), 60 µM (p=0.0004), and higher tested concentrations (p= 0.0001). One-way ANOVA followed by Tukey's post hoc test: $F_{9, 23}$= 121.4; n=33. Data shown as mean ± SD.

CUR modulates ecto-enzymes

We found the modulation of ecto-enzymes by CUR at the concentration of 40 µM and by VIN at 20 nM. Figure 2 displays the results of ATP hydrolysis (Figure 2A), ADP (Figure 2B), AMP (Figure 2C), and ADA activity (Figure 2D). There were no differences in ATP or AMP hydrolysis (Figures 2A and 2C). However, we found different outcomes for the removal of the ADP substrate (one-way ANOVA: F3,4=61.09)(Figure 2B). Whereas VIN increased the hydrolysis of the substrate (p=0.0183), CUR decreased its decomposition (p=0.0042). Tukey's post hoc test indicated a significant difference between the CUR and VIN groups, and between CUR and CUR+VIN groups. Regarding ADA activity (one-way ANOVA: F3,6=8.6,), VIN (p=0.0140) and CUR+VIN (p=0.0123) decreased its activity (Figure 2D) compared to the control, and possibly increased adenosine concentrations. Tukey's post hoc test did not show any significant differences between the other groups.

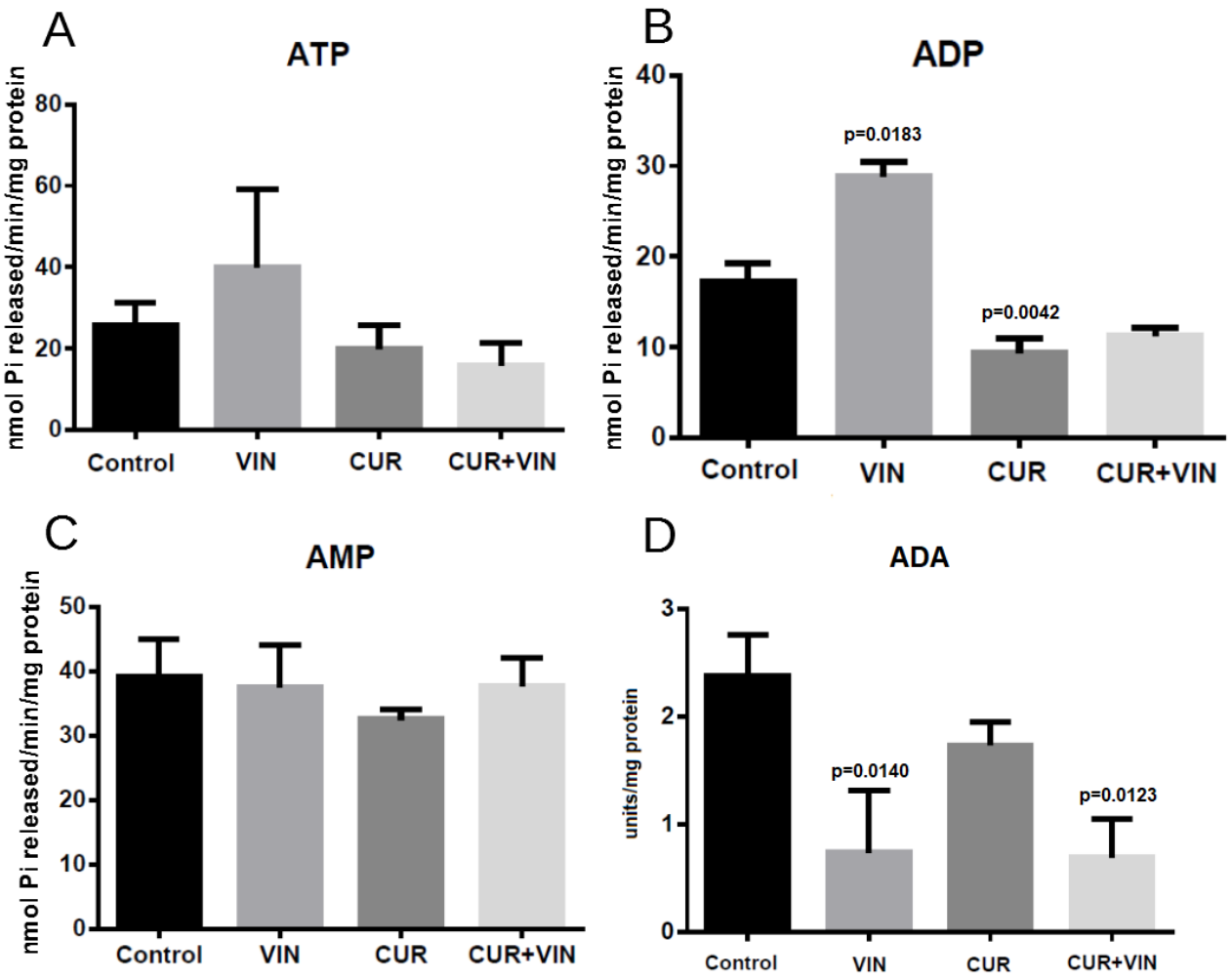

Figure 2. ATP (A), ADP (B) and AMP (C) hydrolysis by E-NTPDase and E-5'- nucleotidase and adenosine deamination (D) in SK-MEL-28 cells after 24 h treatment with, 20 nM of vinblastine (VIN), 40 µM curcumin (CUR), or a combination of both (CUR+VIN). Enzyme-specific activities reported as nmol of Pi released/min/mg of protein. Enzyme activities are reported as U/mg protein. Variables were expressed as mean ± standard error of the mean (SD). Data are shown as mean ± SD. One-way ANOVA followed by Tukey's post hoc test (A – n=9; B – n=8; C – n=9; D – n=10).

CUR produces ROS in SK-MEL-28 cells

Results from oxidative assays (Figure 3) indicate that CAT activity was increased ($F_{3,32}=3.431$, $p=0.0185$) in VIN-treated cells (Figure 3A), while no statistical difference was observed in the other groups. SOD activity was increased ($F_{3,25}=7.496$, $p=0.0385$) in CUR-treated melanoma cells (Figure 3B) compared to control cells, while no statistical difference was detected in the other groups. Tukey's post hoc test showed a significant difference between the CUR and VIN groups and between CUR and CUR+VIN groups.

An increase ($F_{3,38}=5.065$, $p=0.0218$) in protein damage of VIN-treated cells, assessed by the carbonylation assay, was detected (Figure 3C). Tukey's post-hoc test showed a statistically significant difference between the VIN and CUR+VIN groups ($p=0.0042$). No statistical differences were detected in the TBARS assay (Figure 3D).

There was an increase in the detection of ROS ($F_{3,14}=44.9$) in the VIN, CUR, and CUR+VIN groups (Figure 3E). The highest increase was detected in the VIN group ($p=0.001$), followed by the CUR ($p=0.001$) (Figures 3F, G, and H) and CUR+VIN groups ($p=0.0028$). Tukey's post hoc test showed a significant difference between the VIN and CUR groups and between VIN and CUR+VIN groups. The CUR+VIN group also showed a significant difference compared to the CUR group.

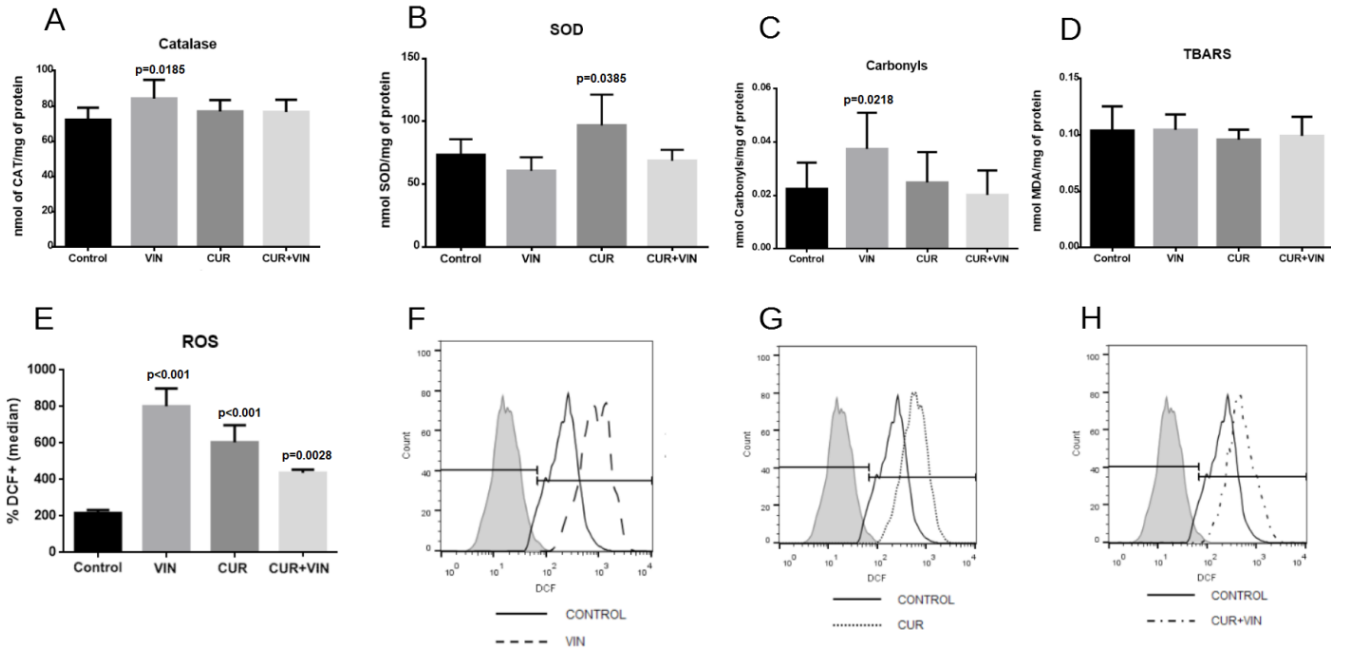

Figure 3. (a) Catalase (CAT) and (b) SOD activities in SK-MEL-28 cells after exposure to 20 nM of vinblastine (VIN), 40 µM curcumin (CUR), or a combination of both for 24 h. (c) Protein carbonylation, (d) TBARS, and (e) ROS production after 24 h treatment with, 20 nM of vinblastine (VIN), 40 µM curcumin (CUR), and a combination of both (CUR+VIN). Histogram presentation of gated cells in DCFH assay of ROS quantification by flow cytometry in cells treated with CUR (F), VIN (G), and CUR+VIN (H) for 24 h. The continuous line represents untreated SK-MEL-28 cells (control). Data are shown as mean ± SD, representative for three independent experiments. One-way ANOVA followed by Tukey's post hoc test.

DISCUSSION

Melanoma is a multifactorial disease associated with increased oxidative stress and inflammation. In vivo and in vitro studies have demonstrated the anti-tumor effects of curcumin against a wide variety of cancers, including colon, duodenum, esophagus, stomach, liver, breast, leukemia, oral cavity, and prostate cancer [29]. We investigated oxidative stress and the activity of ecto-enzymes in SK-MEL-28 cells treated and untreated with CUR, VIN, or a combination of both.

CUR, VIN, and CUR+VIN increased ROS generation in SK-MEL-28 cells. Even though a marked increase in ROS was detected, there was no alteration in lipid and protein damage indicators in cells except those treated with VIN, whose protein carbonylation increased significantly (Figure 3C, p=0.0218). Protein carbonylation in neuroblastoma cells treated with garlic compounds has been previously described [30] to be associated with increased ROS levels after treatment. The cytoskeleton is a dynamic component of the cell as it is involved in the maintenance of cell shape, intracellular trafficking, cell division, cell migration, and adhesion. It is one of the preferential targets of ROS because of the relatively high abundance of oxidizable residues in its protein constituents. Since vinblastine is known for its antitumor interaction with cellular tubulin [19], we hypothesized that this protein interaction was involved in the detected increase in protein carbonylation levels.

ROS are important molecules involved in cell proliferation and differentiation, but high levels of ROS may damage cellular components [31], promote inflammation, and cause apoptosis. Curcumin has been studied as an adjuvant therapy for the treatment of cancer, since it induces apoptosis, generates ROS, and causes oxidative stress in cancer cells. Oxidative stress leads to inflammation in the tumor microenvironment, thus interfering with purinergic signaling.

Cancer cells have increased levels of ROS compared to those in healthy cells [32]. Curcumin exerts its antitumor effects by scavenging or generating ROS, diminishing ROS at low concentrations, or increasing ROS at high concentrations [33]. In our work, CUR increased ROS generation and increased apoptosis (manuscript in preparation). CUR increases SOD activity, a key enzyme of ROS metabolism [34].CUR has already been tested on healthy cells with no toxic effects [35]. VIN increased CAT activity. Higher activities of oxidative stress-related metabolic enzymes have been reported in melanoma cells [36].

The hydrolysis of ATP, ADP, and AMP is increased in the plasma of patients with prostate cancer when compared to healthy patients, thus producing adenosine [37], causing immunosuppression [38], cancer cell

proliferation, and the apoptosis of healthy stromal cells, favoring angiogenesis [39]. VIN increased ADP hydrolysis (Figure 2B), supposedly diminishing intracellular ADP and increasing AMP, and therefore adenosine. The possible increase in adenosine was reinforced by a decrease in the ADA activity in VIN-treatment groups (Figure 2D, alone or in combination with CUR). An increase in extracellular concentrations of adenosine is linked to tumor progression, chemotaxis, migration, invasion, and metastasis [40]. As paradoxical as it seems, since we evaluated the effects over 24 h, further studies are warranted to evaluate the effect over longer periods. CUR, on the other hand, reduced the hydrolysis of ADP (Figure 2B), disrupting the purinergic signaling cascade and reducing the concentration of AMP (and subsequently adenosine). This would reduce the immunosuppressive tumor microenvironment.

High ATP hydrolysis might lead to the formation of large amounts of adenosine, thereby leading to immunosuppression [41]. In our study, VIN increased the hydrolysis of ADP and reduced ADA activity. Thus, VIN might have increased adenosine in the tumor microenvironment and favored immunosuppression.

Long-lasting elevated extracellular ATP levels have been detected after surgical removal of melanoma, resulting in the development of a chronic inflammatory microenvironment [39]. In our work, the use of CUR disrupted the purinergic cascade, reducing the hydrolysis of ADP, and possibly reducing adenosine concentrations in the tumor microenvironment.

The accumulation of ROS can alter mitochondrial membrane potential and cause apoptosis [43]. Although no changes in cell damage markers (MDA and carbonyl proteins) were detected, it is possible that the increase in ROS caused inflammation and impaired purinergic signaling in cells treated with CUR. Other studies have detected alterations in oxidative markers using CUR [42,43]. Since we only evaluated CUR for 24h, we suppose that there was not enough time to reach a detectable alteration of markers.

In conclusion, CUR increased ROS generation in melanoma cells and disrupted the purinergic signaling cascade. This increase in ROS levels may lead to oxidative stress and inflammation. In addition, VIN increased ROS production and adenosine accumulation in cells. CUR has important anti-immunosuppressive effects and might be a promising candidate for the treatment of melanoma, a cancer with high incidence and lethality. Further studies are warranted to evaluate the dynamics of these enzymes over longer periods of time.

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