In vitro effects of L-kynurenine and quinolinic acid on adhesion, migration and apoptosis in B16 F10 melanoma cells

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Abstract:

Introduction: The inhibition of melanoma adhesion through adhesion molecules, such as integrins and E-cadherin, may represent a promising strategy for managing melanoma metastasis. Compounds, namely L-kynurenine (L-kyn) and quinolinic acid (Quin), previously displayed anti-cancer effects at half-maximal inhibitory concentration (IC_{50}) against B16 F10 melanoma cells *in vitro*. However, the role of these compounds in B16 F10 melanoma cell adhesion, migration and apoptosis remain unknown.

Methods: Post-exposure to the compounds, flow cytometry was used to analyse the expression of very late antigen-5 (VLA-5), E-cadherin and cleaved caspase-3 in B16 F10 melanoma and RAW 264.7 murine macrophage cells. An adhesion assay was used to quantify the adhesion of both cell lines to vitronectin. A scratch migration assay was used to measure the possible inhibition of cell migration in B16 F10 cells in response to L-kyn and Quin.

Results: In both B16 F10 and RAW 264.7 cells, neither L-kyn nor Quin induced significant effects on VLA-5 expression or cell adhesion to vitronectin. In B16 F10 cells, both L-kyn and Quin elevated E-cadherin expression and displayed a trend of suppressed migration. However, only L-kyn elevated E-cadherin in RAW 264.7 cells. L-kyn induced apoptosis by elevating cleaved caspase-3 expression in both cell lines.

Conclusion: L-kyn and Quin demonstrated promising antimetastatic effects in their ability to elevate E-cadherin expression and induce apoptosis in B16 F10 melanoma cells. However, these effects did not occur in response to vitronectin or VLA-5 integrin alterations. Furthermore, it cannot be excluded that L-kyn also induced apoptosis in RAW 264.7 cells. As such, these effects should be confirmed in additional control cell lines and substantiated with *in vivo* models.

Keywords: melanoma, kynurenine metabolites, L-kynurenine, quinolinic acid, adhesion, apoptosis

1. Introduction:

Melanoma is a highly metastatic malignancy, accounting for more than 80% of skin cancer-related deaths.[1] With an incidence that continues to rise,[2] melanoma remains the leading cause of death from skin cancer.[3] To date, melanoma has evaded all attempts at treatment, which can be ascribed to its metastatic nature.[4] Metastasis encompasses a series of stages, including invasion, migration, intravasation and extravasation of cancer cells.[5] Importantly, all stages of metastasis involve cell adhesion,[6-8] which is mediated by cell adhesion molecules, such as 1) integrins and 2) E-cadherin.

Firstly, integrin-mediated cell adhesion is activated by bidirectional integrin signal transmission from the “inside-out” (intracellular integrin domain to the extracellular integrin domain) and “outside-in” (intracellular integrin domain to the extracellular integrin domain).[6, 7] Integrins, namely, very late antigen-4 (VLA-4; α4β1) and VLA-5 (α5β1), have proven their involvement in melanoma progression and have been associated with poor patient prognosis and metastasis.[6] Metastasis is a known consequence of cell adhesion, as cell-cell and cell-extracellular matrix (ECM) adhesion is crucial for cell survival and proliferation.[9] In contrast, the loss of cell adhesion activates signalling cascades, which lead to the activation of the effector caspase, namely caspase-3 and induces a type of apoptotic cell death known as anoikis,[10] a type of apoptosis induced by detachment from the ECM and surrounding cells.[11]

Secondly, E-cadherin, an intracellular adhesion molecule, plays a crucial role in cancer metastasis through its involvement with cell-cell adhesion and its role in allowing cells to dissociate from the primary tumour. This leads to tumour cell invasion and subsequent metastasis by migrating to distant sites. As such, it is clear that E-cadherin is involved in metastatic parameters, including tumour cell-cell adhesion and cell migration.[12] The latter involves cell-cell and cell-ECM interplay via changes in adhesion molecules. In metastatic melanoma, changes in adhesion molecules contribute to cancer cell motility in order to metastasise (spread) from the primary site to a secondary organ.[4] During metastasis, directed cell migration is facilitated by the intricate interplay between cadherins and integrins, which may consequently also determine cell fate,[13] supporting the notion that loss of cell-cell adhesion or cell-ECM adhesion can lead to cell death.

Molecules in signalling pathways, such as extracellular-signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K), contributing to cancer metastasis, may be activated by the

kynurenine pathway. The literature describes that these kynurenine pathway-mediated signalling pathways are involved in various cellular processes contributing to metastasis, such as migration, apoptosis, survival, and proliferation.[14] Additionally, various kynurenine metabolites act as biologically active substances and their potential anti-cancer properties against melanoma have been well-described.[6]

Recent evidence reported that the exogenous administration of kynurenine metabolites, namely L-kynurenine (L-kyn) and quinolinic acid (Quin) induced cell death in B16 F10 melanoma cells at half-maximal inhibitory concentration (IC_{50}) after 48 hours *in vitro*[15] through ERK1/2 activation inhibition.[16] Therefore, it is suggested that these metabolites (L-kyn and Quin) may also exert an effect on adhesion, migration and apoptosis, through the interplay between E-cadherin and integrins in B16 F10 melanoma cells. Due to limited research on adhesion and migration effects of these tryptophan metabolites (L-kyn and Quin), this study aimed to investigate the ability of these compounds to determine the impact on cell adhesion, migration, VLA-5, E-cadherin and cleaved caspase-3. Previously, the concentration at which these compounds exhibited cytotoxicity (50% cell death), was reported. This study aimed to investigate the ability of these compounds at the previously reported concentrations to determine the impact on cell adhesion, migration, VLA-5, E-cadherin and cleaved caspase-3.

2. Methods:

2.1 Cell lines

2.1.1 Melanoma

The melanoma (B16 F10) cell line, was purchased from the American Type Culture Collection B16 F10 (ATCC® CRL-6475™). This cell line was previously used to determine the IC_{50} values, used in this study [15] between passages 4-12.

2.1.2 Non-cancerous macrophage cells

The RAW 264.7 cell line, purchased from CELLONEX, South Africa was used as the control cell line as it is a non-cancerous, macrophage-like cell line. The justification for the use of this control cell line is that macrophages express all the enzymes in the kynurenine pathway, including indoleamine 2,3- dioxygenase (IDO), which is the rate-limiting step.[17] This cell line was used between passages 6-20.

2.2. General culture maintenance and sample preparation

Cells were cultured and maintained at 37°C with 5% CO_2 in a Forma Scientific water-jacketed incubator in sterile culture flasks. Completed culture medium (CCM), comprising of Dulbecco's modified essential medium (DMEM), 10% fetal calf serum (FCS) and 1% antibiotics

(amphotericin/penicillin/streptomycin) was used for cell culture. All compounds, including L-kyn (1.74 mM), Quin (8.23 mM) and nocodazole (NOC) (1.30 mM) were prepared at IC_{50} values obtained in B16 F10 cells in a previous study conducted by Basson *et al*.[15] NOC was used as the positive control. L-kyn, Quin and NOC were dissolved in ≤0.1% volume per volume (v/v) dimethyl sulfoxide. L-kyn and Quin were diluted in 0.1 M phosphate-buffered saline (PBS) and NOC in ddH_2O. The diluent for each compound was used as a vehicle control for experiments.

2.3. Cell adhesion assay

A cell adhesion assay was used to assess tumour cell adherence to an ECM protein (specifically vitronectin) before and after treatment with L-kyn and Quin. Upon exposure to various compounds, the cellular metabolic activity of the cells was measured with a methylthiazolydiphenyl tetrazolium bromide (MTT) assay, where metabolically active cells (containing NAD(P)H-dependent oxidoreductase enzymes) reduce yellow tetrazolium salt to purple formazan crystals. Non-adherent cells undergoing cell death will, therefore, not be metabolically active and not reduce yellow tetrazolium salt to purple formazan crystals.[18] A cell adhesion assay was conducted in accordance with the literature and outlined below.[19]

Vitronectin, at 5 µg/mL, was prepared in ddH_2O and used to coat wells in 96-well plates at 20 µL per well. The 96-well plates were stored at 4°C for 24 hours prior to seeding. Cells were seeded in 24-well plates, where B16 F10 cells were seeded in a volume of 0.45 mL at a concentration of 15×10^4 cells per mL (6.75×10^4 cells/well) and RAW 264.7 cells were seeded in a volume of 0.45 mL at a concentration of 20×10^4 cells per mL (9×10^4 cells/well). Cells were incubated overnight, whereafter cells were exposed to L-kyn, Quin and NOC at IC_{50} for 48 hours. After 48 hours, cells were harvested and centrifuged at 504 xg for five minutes. Cells were washed twice in 1 mL CCM and centrifuged at 504 xg for five minutes between washes. Thereafter, the cells were resuspended in serum-free medium (SFM) for 4 hours. After 4 hours, cells were centrifuged for 3 minutes, and pellets were resuspended in 0.1% bovine serum albumin (BSA) (diluted in SFM). Cells were then seeded in 96-well plates, where B16 F10 cells were seeded at 5 000 cells per well and RAW 264.7 cells were seeded at 10 000 cells per well followed by incubation for 3 hours at 37°C and 5% CO_2 in a humidified incubator. After 3 hours, MTT (10 µL 0.005 g/mL 0.1 M PBS) was added to each well and incubated for an additional 2 hours. After 2 hours, 100 µL of 100% dimethyl sulfoxide (DMSO) was added to each well, to determine cell adherence and viability. Absorbance was measured at 570 nm using the EPOCH BIOTEK spectrophotometric plate reader (Winooski, Vermont, US).

2.4. Flow cytometry

B16 F10 and RAW 264.7 cells were seeded in a volume of 9 mL at a concentration of 70 x 10^4 in T75cm^2 tissue culture flasks (6.3 x 10^6 cells/flask). After allowing for attachment, cells were exposed to L-kyn and Quin respectively. Cells were harvested and centrifuged at 504 xg for five minutes. The supernatant was discarded, and the pellet was washed twice with ice-cold PBS. The supernatant was discarded and ice-cold 70% methanol (v/v) was added to the pellet in a dropwise manner while the sample was vortexed. Samples were stored at -20°C until further analysis. Upon staining, cells were vortexed and analysed on the Flow CheckTM Pro (Beckman Coulter, Miami, USA) attached to an air-cooled argon laser.

Flow cytometry analyses involved the use of unstained control cells treated with PBS to set positive gates. A minimum of 15 000 cells were analysed using Kaluza C data analysis software (Version 1.1.00003.20057 Beckman Coulter). Additionally, the analyses involved the exclusion of debris and doublets. At least two repeats (n=2) (mean ± standard error of mean) were conducted for flow cytometric studies. Data, as provided in the supplementary files is presented as a composite univariate histogram. This data is indicated as overlay histograms with unstained cells and stained control and treated cells for B16 F10 and RAW 264.7 cells, respectively. Statistically, L-kyn and Quin were compared to the PBS:CCM control cells and NOC-treated cells were compared to the ddH_2O:CCM control cells.

2.4.1. Protein expression quantification of VLA-5

After sample preparation, explained in section 2.4 above, the samples were washed once with a 0.5% BSA/PBS buffer. The supernatant was discarded and 100 µL of mouse integrin alpha 5/CD49e Alexa fluor 488, which is a conjugated antibody, at a concentration of 1 µg/mL in 0.5% BSA/PBS buffer was added to 1 000 000 cells per sample, as per the manufacturer's instructions. Samples were incubated for 30 minutes at room temperature in the dark after which cells were resuspended in 1 mL 0.5% BSA/PBS buffer.

2.4.2. Apoptosis detection with Caspase-3

After sample preparation explained in section 2.4 the samples were washed once with a 0.5% BSA/PBS buffer. The supernatant was discarded and 0.25 µg mouse-cleaved anti-caspase-3 (asp 175) 405 nm at a concentration of 0.5 mg/mL in sterile PBS was added to 1 000 000 cells per sample, as per the manufacturer's instructions. Samples were incubated for 40 minutes at 2-8 °C, centrifuged and then resuspended in 1 mL 0.5% (w/v) BSA/PBS buffer.

2.4.3. Protein expression quantification of E-cadherin

After sample preparation explained in section 2.4 the samples were washed once with a 0.5% BSA/PBS buffer. The supernatant was discarded and 0.5 µg mouse E-cadherin Alexa Fluor 405- conjugated antibody at a concentration of 0.2 mg/mL in sterile PBS/BSA was added to 1 000 000 cells per sample, as per the manufacturer's instructions. Samples were incubated for 30 minutes at room temperature in the dark. After 30 minutes, cells were centrifuged, resuspended in 1 mL 0.5% (w/v) BSA in PBS buffer.

2.5. Scratch migration assay

B16 F10 cells were seeded in a volume of 0.45 mL at a concentration of 20×10^4 cells per mL (9×10^4 cells/well) in 24-well plates. The scratch migration assay was only performed in the adherent cell line (B16 F10 cells) and not in the semi-adherent cell line (RAW 264.7 cells), as this assay is recommended for adherent cell lines.[20] The cells were incubated at 37°C and 5% CO_2 in a humified atmosphere for 24 hours to allow for attachment. The CCM was removed, and a vertical scratch was made with a sterile 100 µL pipette tip across the centre of the well. A second scratch was made perpendicular to the first to form a cross. To remove detached cells, the wells were carefully rinsed twice with medium.

Cells were then exposed to L-kyn and Quin (at concentrations ten times lower than the IC_{50}) where L-kyn was at 0.174 mM, Quin at 0.823 mM and NOC at 0.13 mM to determine whether these compounds induce anti-migratory activity at a non-cytotoxic concentration. Controls for this experiment included cells treated with CCM:PBS and CCM:ddH_2O. Positive controls included cells exposed to NOC (an anti-migratory drug).[21] The doubling time of B16 F10 melanoma cells has been reported as 20.1 hours.[22] In order to ensure that the data captures the compounds' effect on migration instead of proliferation, images were captured at 0-, 6-, 12 and 18 hours using an Axiovert 40 CFL inverted transmitted-light incident-light fluorescent microscope (Zeiss, Oberkochen, Germany). The gap of each scratch was quantitatively evaluated using Image J software developed by the National Institutes of Health (Bethesda, Maryland, United States of America).

2.6. Compliance with Ethical Standards

The ethical consent for this study was obtained from the University of Pretoria, Faculty of Health Science, Research Ethics Committee (reference number: 405/2020).

2.7. Statistics

Experiments were done 3 times in triplicate and checked for normality using the Shapiro-Wilks test and further tested for significant differences using one-way ANOVA with the Tukey test (if

data was parametric) or the Kruskal-Wallis ANOVA with the Dunn test (if data was non-parametric). Flow cytometry experiments were conducted twice. All quantitative data is represented as mean ± standard error of mean (SEM). All data was checked for significance and $p \leq 0.05$ was considered significant.

3. Results:

3.1. Cell adhesion

After the treatment of B16 F10 cells with L-kyn and Quin at calculated IC_{50} values, the average percentage of cell adhesion to vitronectin was 96.44% for L-kyn-treated cells, 89.44% for Quin-treated cells and 104.69% for NOC-treated cells (Figure 1). However, no statistically significant differences in adhesion of B16 F10 cells to vitronectin were observed. In RAW 264.7 cells, the average percentage of cell adhesion to vitronectin was 122.94% for L-kyn-treated cells, 100.57% for Quin-treated cells and 125.99% for NOC-treated cells (Figure 1). However, no statistically significant differences in adhesion of RAW 264.7 cells to vitronectin were observed.

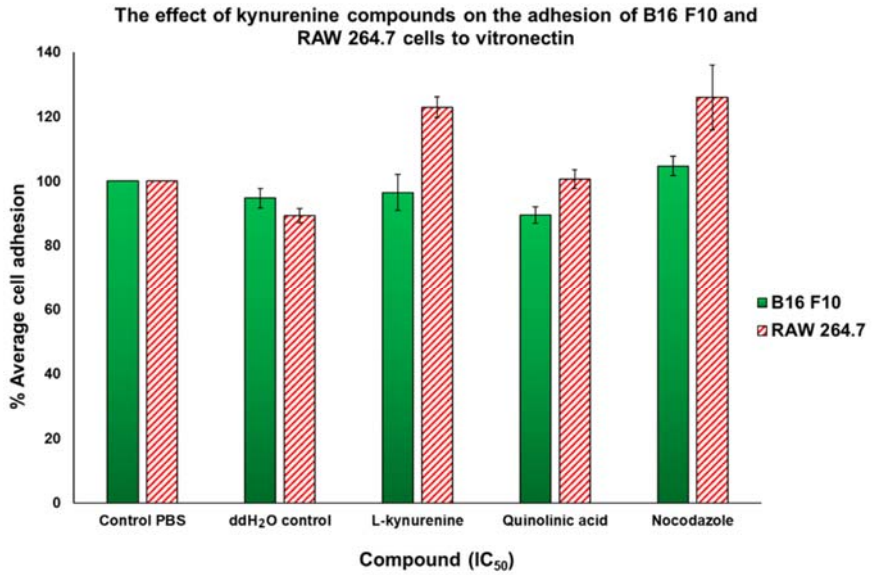

Figure 1: L-kyn and Quin at IC_{50} on the adhesion of B16 F10 and RAW 264.7 cells to vitronectin after 48 hours for control cells treated with PBS, control cells treated with ddH_2O, L-kynurenine-treated cells at 1.74 mM, quinolinic acid-treated cells at 8.23 mM and positive control cells treated with NOC at 1.30 mM. Adhesion values are expressed as the percentage of viable cells relative to CCM: PBS-treated control samples of at least 3 experimental repeats, with the standard error of mean (SEM) indicated by the error bars.

3.2. VLA-5 quantification

The ddH_2O-treated control cells did not display any significant effects compared to the PBS-treated control cells in B16 F10 or RAW 264.7 cells. Neither L-kyn, Quin nor NOC significantly affected the expression of VLA-5 in the B16 F10 or RAW 264.7 cell line (Figure 2).

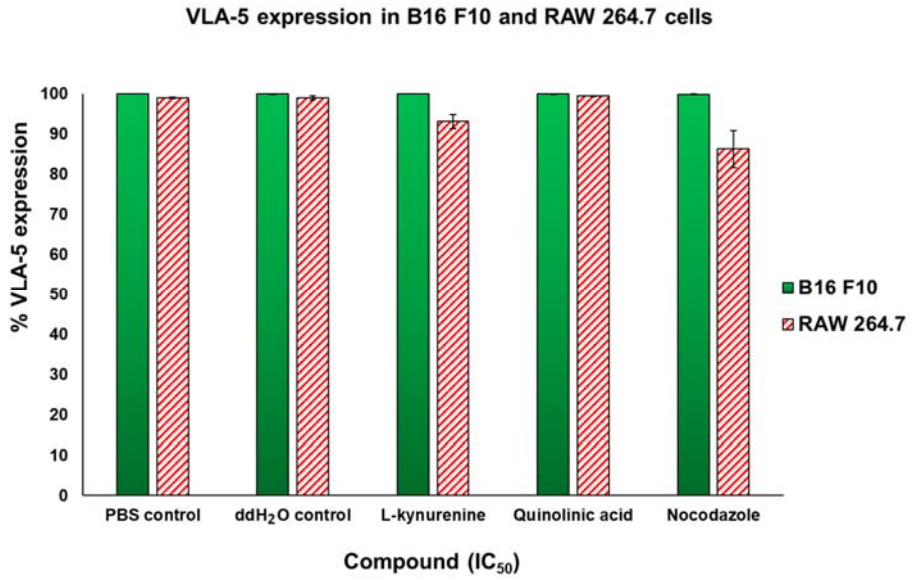

Figure 2: VLA-5 expression in B16 F10 and RAW 264.7 cells after 48 hours for control cells treated with PBS, control cells treated with ddH_2O, L-kynurenine-treated cells at 1.74 mM, quinolinic acid-treated cells at 8.23 mM and positive control cells treated with NOC at 1.30 mM. The bar graphs represent the average of at least 2 experimental repeats, with the standard error of mean (SEM) indicated by the error bars.

3.3. Caspase-3

For the B16 F10 cell line, L-kyn exposure significantly increased cleaved caspase-3 (99.99 ± 0.01). Quin non-significantly decreased cleaved caspase-3 (1.95 ± 0.15). NOC exposure significantly decreased cleaved caspase-3 (2.59 ± 0.81). In the RAW 264.7 cell line, L-kyn exposure significantly increased cleaved caspase-3 (99.89 ± 0.11) (Figure 3).

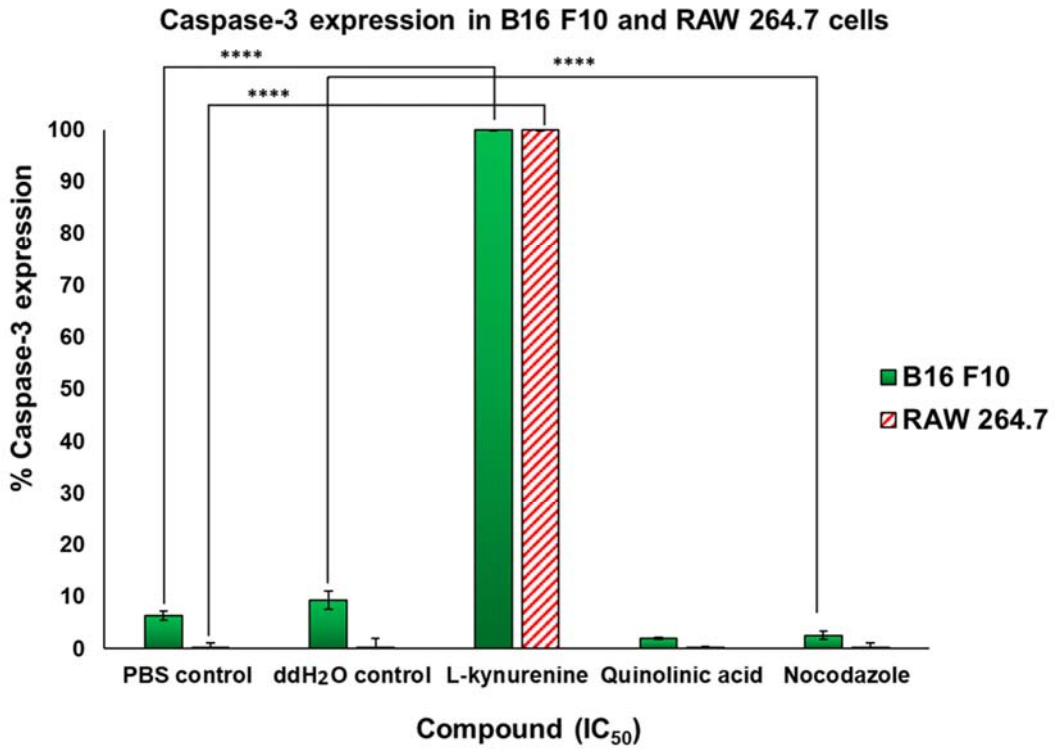

Figure 3: Cleaved caspase-3 in B16 F10 and RAW 264.7 cells after 48 hours for control cells treated with PBS, control cells treated with ddH_2O, L-kynurenine-treated cells at 1.74 mM, quinolinic acid-treated cells at 8.23 mM, and positive control cells treated with NOC at 1.30 mM. The bar graphs represent the average of at least 2 experimental repeats, with the standard error of mean (SEM) indicated by the error bars. **** $p \leq 0.0001$ indicates significant difference when compared to the control treated with PBS or ddH_2O.

3.4. E-cadherin

For the B16 F10 cell line, L-kyn exposure (99.59 ± 0.017), as well as Quin exposure (95.79 ± 1.07), significantly increased E-cadherin expression. Additionally, exposure to the positive control NOC increased E-cadherin expression (92.51 ± 1.02). In RAW 264.7 cells, L-kyn exposure significantly induced E-cadherin expression (77.26 ± 5.88). (Figure 4).

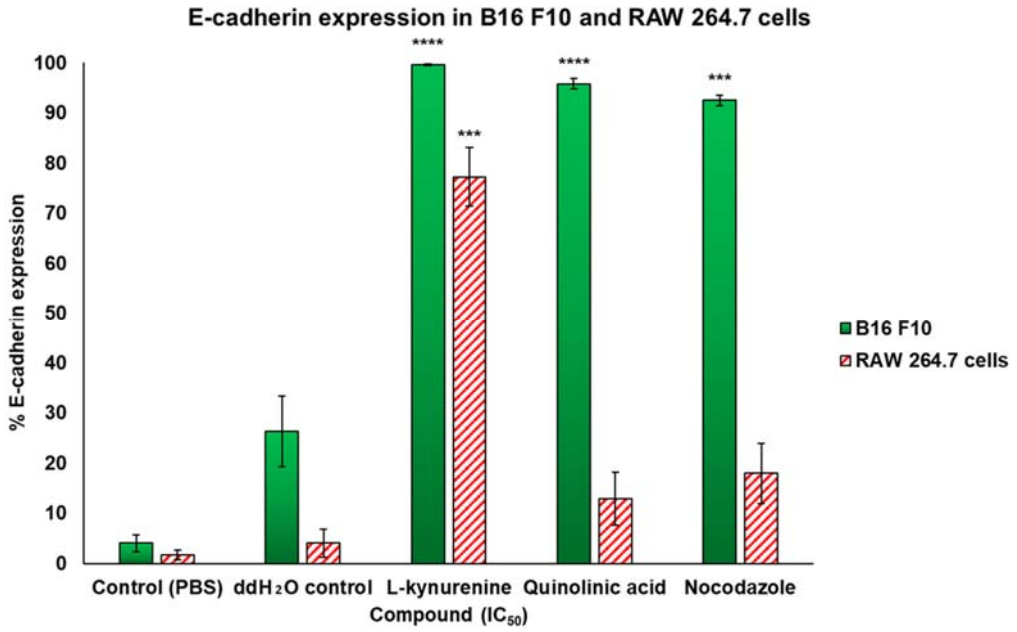

Figure 4: E-cadherin expression in B16 F10 and RAW 264.7 cells after 48 hours for control cells treated with PBS, control cells treated with ddH_2O, L-kynurenine-treated cells at 1.74 mM, quinolinic acid-treated cells at 8.23 mM, and positive control cells treated with NOC at 1.30 mM. The bar graphs represent the average of at least 2 experimental repeats, with the standard error of mean (SEM) indicated by the error bars. ***$p \leq .001$; **** $p \leq 0.0001$ indicates significant difference when compared to the control treated with PBS or ddH_2O.

3.5. Migration

Figure 5 illustrates the percentage wound closure over time, comparing the effects of each compound at the initial time point (0 hours). Although non-significant, L-kyn, Quin and NOC-treated cells displayed a trend of reduced cell motility.

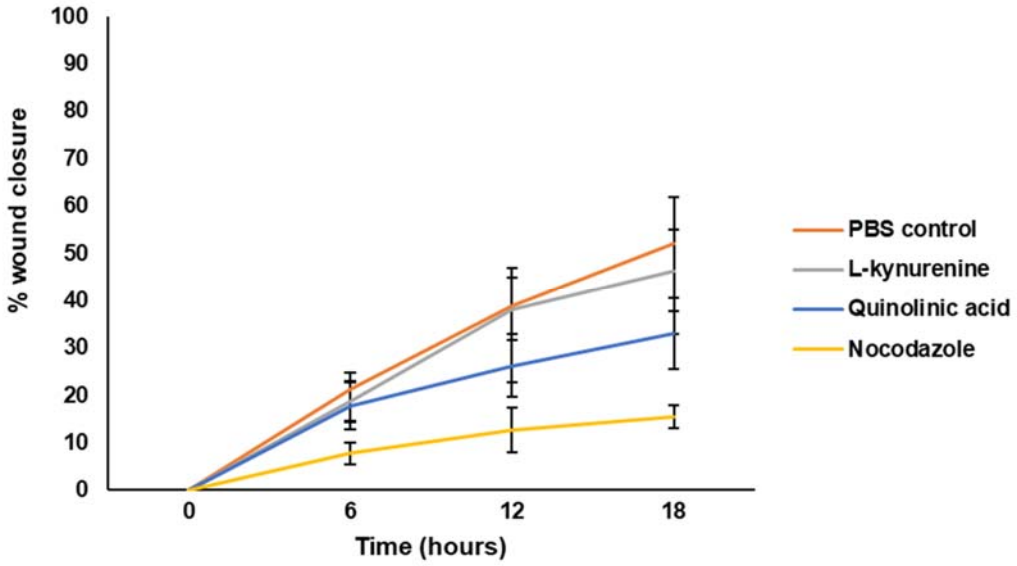

Figure 5: B16 F10 cell migration after exposure to L-kyn at 0.174 mM and Quin at 0.823 mM after 6, 12 and 18 hours. The bar graphs represent the average of at least 3 experimental repeats, with the standard error of mean (SEM) indicated by the error bars.

3. Discussion:

Recent findings have demonstrated that the exogenous administration of kynurenine metabolites, specifically L-kyn, Quin and kynurenic acid (KA), induces cytotoxicity in RAW 264.7 macrophage, HaCat keratinocytes and B16 F10 melanoma cells at IC_{50}.[15] Mechanistically, it has been reported that L-kyn and Quin exhibited the aforementioned effects by inducing cell death (apoptosis and necrosis), alterations in cell cycle progression and the inhibition of ERK1/2 activation.[16] The inhibition of the MEK/ERK signalling pathway may occur as a result of the inhibition of cell adhesion. As such, cell adhesion molecules, which provide protection against cell death, may be downregulated. This may result in cell death, such as autophagy and apoptosis.[23] Considering the possible intricate interplay between ERK1/2, cell survival and cell adhesion molecules (E-cadherin and integrins) within intracellular signalling pathways, it was hypothesised that L-kyn and Quin may also exert effects on cellular adhesion to ECM proteins (specifically vitronectin), migration, VLA-5 integrin expression, E-cadherin levels, and cleaved caspase-3 activity in B16 F10 melanoma cells.

Investigating the effects of L-kyn and Quin on these cellular processes in B16 F10 melanoma cells holds significant importance. Prior *in vitro* studies have pointed towards the antitumorigenic and -metastatic effects of L-kyn, possibly through aryl hydrocarbon receptor (Ahr) activation in different cancer types, such as colon cancer cells and human glioma cells.[24] Contradictory to these results, other studies have indicated that Ahr activation may also have

pro-tumorigenic and -metastatic effects. This was reflected in previous studies where L-kyn induced the proliferation of HCT 116 colon cancer cells *in vitro.*[24] Similarly, Quin, an N-methyl-D-aspartate (NMDA) agonist [25] has been shown to induce cell death in B16 F10 melanoma cells,[15] but enhanced glioblastoma cell survival.[26] Given this dichotomy in findings, an in-depth investigation of the effects of L-kyn (an Ahr agonist) and Quin (an NMDA agonist) on B16 F10 melanoma cells *in vitro* is warranted. From these conflicting perspectives, it can be inferred that L-kyn and Quin likely play a role in tumour cell adhesion, migration, apoptosis and proliferation. However, based on the abovementioned contradicting results from previous studies, L-kyn and Quin could possibly elicit non-beneficial effects on these metastatic parameters.

In order to investigate the effects of L-kyn and Quin on B16 F10 melanoma cell adhesion, it is essential to take the ECM into account, as cancer progression involves complex signalling between cancer cells and the surrounding ECM.[6] Cell-ECM interactions are typically associated with integrins that bind ECM constituents such as fibronectin, laminin, collagen, tenascin, thrombospondin and vitronectin.[27] Vitronectin is a multifunctional glycoprotein anchored to the ECM via its collagen-binding domain and its glycosaminoglycan (GAG) binding domain to promote cell adhesion and migration.[28] This study demonstrated that neither L-kyn nor Quin influenced the adhesion of B16 F10 or RAW 264.7 cells to vitronectin. However, the authors recommend that future studies should include other ECM proteins to confirm the effect of L-kyn and Quin on B16 F10 melanoma cell adhesion.

Additionally, the interaction of vitronectin with integrins is implicated in cell proliferation.[28] Cell-ECM-detachment disrupts integrins and ultimately results in apoptosis.[29] Integrin-ECM interactions, therefore, play a crucial role in controlling apoptotic and cell survival signalling.[30] It is well-known that integrin expression can vary considerably between normal and tumour cells, as the expression levels can be highly upregulated in some tumours, which may promote tumour progression and metastasis.[28] Therefore, VLA-5 expression, which are one of the integrins expressed in melanoma cells, was investigated.[6] These integrins have been associated with metastasis and poor patient prognosis.[6] This current study demonstrated that VLA-5 was expressed in B16 F10 cells. However, neither L-kyn nor Quin resulted in a significant decrease of VLA-5 expression in B16 F10 or RAW 264.7 cells. Collectively, these findings suggest that the inhibition of proliferation, previously demonstrated in response to L-kyn and Quin at IC_{50} values in B16 F10, did not occur due to alterations in vitronectin or VLA-5, and that other mechanisms should be investigated.

As such, E-cadherin was investigated due to its involvement in tumour cell adhesion and cell fate.[12, 13 31] To the best of our knowledge, this is the first study to investigate the effects of E-cadherin upon the exogenous treatment of cancer cells with L-kyn and Quin at their respective IC_{50}. This study demonstrates that L-kyn and Quin significantly elevated E-cadherin expression levels in B16 F10 melanoma cells, which could be an indication of the alteration of complex intracellular signalling pathways that promote metastasis.[32] In support of this statement, it has recently been reported that ERK1/2 was significantly suppressed in response to L-kyn and Quin treatment,[16] highlighting its potential effects on melanoma survival and adhesion. Additionally, E-cadherin regulates epithelial homeostasis by sensitising cells to anoikis through various mechanisms, including β-catenin/Wnt signalling, pro-anoikis signalling via p14Arf, apoptotic signalling through the extrinsic pathway, and activation of the Hippo pathway..[33] Therefore, this study investigated apoptosis by employing caspase-3 quantification to confirm the loss of cell adhesion and apoptotic cell death.[10] Cleaved caspase-3 is a marker of apoptosis,[34, 35] which is an important mechanism of cell survival and chemoresistance in various cancer cells, to induce cell death.[36]

This current study demonstrated that L-kyn, but not Quin induced apoptosis in B16 F10 and RAW 264.7 cells by significantly upregulating caspase-3 (an executioner caspase). These findings correspond to a prior study where L-kyn induced apoptosis (early and late) in B16 F10 melanoma cells as well as necrosis in B16 F10 melanoma cells at IC_{50}. Additionally, the previous study also found that Quin only induced necrosis in RAW 264.7 cells and early apoptosis in B16 F10 melanoma cells,[16] possibly suggesting that the apoptotic effect induced by Quin is insufficient to activate caspase-3 in B16 F10 melanoma cells. Those findings were further supported by transmission electron microscopy images, which revealed signs of both apoptosis and autophagy. Given the potential cross-regulation between overlapping signalling pathways, such as MAP-K/ERK and PI3K/AKT, which are known to induce both apoptosis and autophagy, we sought to confirm apoptosis in the current study. However, these pathways are not mutually exclusive, and therefore cannot be viewed as entirely separate events, as they may co-occur and influence each other.[37-40] As such, it remains necessary to quantitatively assess autophagic activity by examining specific autophagy markers to fully understand the extent of its involvement. In contrast to these findings, Walczak *et al*. demonstrated that L-kyn induced necrosis in melanoma A375 cells.[41] This may indicate that the mode of cell death induced by L-kyn may be cell-line dependent.

In addition to its effect on cell survival and adhesion, the elevated expression of E-cadherin may also be associated with suppressive melanoma cell motility, thereby decreasing melanoma migration.[42] This study found that both L-kyn and Quin displayed a trend of B16

F10 melanoma cell migration inhibition over time (0–18 h) after exposure to concentrations ten times lower than the IC_{50}. Previous research demonstrated the anti-migratory effects of KA in human glioblastoma T98G cells [43] and renal cancer Caki-2 cells.[44] Contrastingly, L-kyn previously induced migration in lung cancer cells *in vitro*, emphasising the contrasting role that this Ahr agonist may play in different cell lines.[24] Importantly, future research should investigate the effects of L-kyn and Quin on control (anti-cancerous cell lines), such as melanocytes or keratinocytes to indicate if these anti-migratory effects are cancer-specific.

4. Conclusion

L-kyn and Quin displayed a trend of inhibited cell migration and induced E-cadherin expression in B16 F10 melanoma cells, which may suggest the ability of these compounds to alter metastasis through the activation of complex signalling pathways. Future research is necessary to investigate the anti-migratory effects of these compounds on control cell lines. Additionally, this study demonstrated that L-kyn induced apoptosis in B16 F10 and RAW 264.7 cells through caspase-3 expression. However, L-kyn and Quin did not induce alterations in the adhesion of B16 F10 cells to vitronectin or VLA-5 integrin expression. These findings warrant further investigations on the exact mechanistic processes governing melanoma cell death induced by L-kyn and Quin, *in vitro* and substantiated further by *in vivo* findings.

5. Limitation

A limitation of this study is that while the migration assay was conducted at concentrations 10 times lower than the IC_{50} to avoid cytotoxic effects and isolate the compounds' anti-migratory activity, the impact of adhesion at these non-cytotoxic concentrations has not been fully explored. Future studies are needed to investigate the effects of these compounds on cell adhesion across various ECM proteins at non-cytotoxic concentrations, enabling a more comprehensive understanding of the relationship between migration and adhesion under consistent experimental conditions.

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7. Conflict of interest statement

The authors declare no conflict of interest.

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