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ORIGINAL ARTICLE

Melatonin and its derivative disrupt cancer stem-like phenotypes of lung cancer cells via AKT downregulation

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Abstract

Cancer stem cells (CSCs), a small subpopulation of tumour cells, have properties of self-renewal and multipotency, which drive cancer progression and resistance to current treatments. Compounds potentially targeting CSCs have been recently developed. This study shows how melatonin, an endogenous hormone synthesised by the pineal gland, and its derivative suppress CSC-like phenotypes of human non-small cell lung cancer (NSCLC) cell lines, H460, H23, and A549. The effects of MLT and its derivative, acetyl melatonin (ACT), on CSC-like phenotypes were investigated using assays for anchorage-independent growth, three-dimensional spheroid formation, scratch wound healing ability, and CSC marker and upstream protein signalling expression. Enriched CSC spheroids were used to confirm the effect of both compounds on lung cancer cells. MLT and ACT inhibited CSC-like behaviours by suppression of colony and spheroid formation in NSCLC cell lines. Their effects on spheroid formation were confirmed in CSC-enriched H460 cells. CSC markers, CD133 and ALDH1A1, were depleted by both compounds. The behaviour and factors associated to epithelial–mesenchymal transition, as indicated by cell migration and the protein vimentin, were also decreased by MLT and ACT. Mechanistically, MLT and ACT decreased the expression of stemness proteins Oct-4, Nanog, and β-catenin by reducing active AKT (phosphorylated AKT). Suppression of the AKT pathway was not mediated through melatonin receptors. This study demonstrates a novel role, and its underlying mechanism, for MLT and its derivative ACT in suppression of CSC-like phenotypes in NSCLC cells, indicating that they are potential candidates for lung cancer treatment.

KEYWORDS
acetyl melatonin, AKT, cancer stem cells, lung cancer, melatonin

1 | INTRODUCTION

A number of studies have reported that a small subpopulation of tumour cells known as cancer stem cells (CSCs) are capable of self-renewal and multipotency. CSCs are a key cause of cancer development, aggressiveness and chemotherapy resistance.[1-4] They can be recognised by stem cell markers such as CD133 and ALDH1A1.[5,6] CSCs also overexpress genes, including Oct-4 and

Nanog, that are known to regulate the stem cell function of multiple cancers.[7-9] The AKT pathway is important for retaining cancer stem-like cells in many types of cancer, including prostate, breast, and lung cancer, and can, therefore, be used as a target of treatment.[10-13] The active form of AKT has been found to phosphorylate glycogen synthase kinase 3β (GSK3β) into inactive phosphorylated GSK3β (p-GSK3β) form; the complex of GSK3β/β-catenin can enhance ubiquitin–proteasome degradation. Without the GSK3β/β-catenin complex, β-catenin level is increased and further moves to the nucleus where stemness genes are transcribed.[14,15] β-catenin is highly expressed in lung cancer cells resistant to chemotherapy. In the absence of β-catenin, a decrease in lung cancer cell proliferation, formation of colony, migration and resistance to chemotherapy are reported.[16,17] In addition, CSC metastasis is regulated by increasing the properties of epithelial-to-mesenchymal transition (EMT). EMT induction in CSCs has been shown to increase stem cell-like phenotypes.[13,18] The increase in EMT markers such as vimentin correlates with enhanced cancer cell migration and invasion.[19]

Melatonin (MLT; Figure 1A) or *N*-acetyl-5-methoxytryptamine, an indolamine, is generally considered a multitasking molecule. It is secreted by the pineal gland to control circadian rhythms.[20] It is used for the management of many symptoms and diseases, including insomnia,[21] migraine,[22] and Parkinson's disease.[23] A number of studies have reported antioxidant, immunomodulatory, anti-inflammatory, and anti-cancer properties for MLT.[20,24-26] Recently, MLT was found to inhibit CSCs in breast, ovarian, brain and colon cancers.[27-31] It decreased the size and number of spheroids in MCF-7 cells, in both spheroid-formation assay and anchorage-independent growth.[29] MLT has a role in inhibiting the survival and invasiveness of breast CSCs as well as in regulating the EMT-related proteins expression.[28] The effect of MLT was investigated in mammospheres consisting of $CD44^+$ cells from human breast cancer cell lines, MCF-7; the viability, migration, and invasion of the mammospheres were reduced by treatment with MLT.[28] The expression of the self-renewal protein Oct-4 and vimentin decreased in the MLT-treated cells. In the SKOV3 ovarian cancer cell line, there was a decrease in the percentage of $CD133^+/CD44^+$ cells in the presence of MLT.[27] Self-renewal and tumorigenicity of glioblastoma stem-like cells (GSCs) of primary GSC cultures were also impaired by MLT treatment.[31] GSCs showed reductions in the rate of proliferation of GSC clonogenicity. Stem cell markers of GSCs (CD133, and Sox2), and upstream signalling protein (phosphorylated AKT), were found to be reduced after treatment with MLT. Indeed, when GSCs were pre-incubated with luzindole, a MLT receptor antagonist, the suppression of CD133 by MLT was impaired. This study indicated that the anti-CSC effect of MLT was regulated via the MLT receptors.[31] MLT mainly acts on the G-protein coupled receptor family, MT1 and MT2.[32] MLT receptors are expressed in various cancer cells, including CSCs.[27,31,33] Previous studies have also shown that the MLT receptors are involved in the anti-proliferation and anti-metastasis of MLT in cancer cells.[33,34] Suppression of AKT by MLT was diminished through MLT receptors in uterine leiomyoma cells.[35] However, the effect of MLT on lung CSCs is largely unknown.

Because of the therapeutic potential of MLT as mentioned above and its widespread use as a nutritional supplement, we have aimed to find its effect on CSCs and the underlying mechanisms of action. However, previous studies on oral administration of MLT have reported low bioavailability (3%–33%) and large inter-subject variability.[36,37] Therefore, novel MLT derivatives that can improve MLT bioavailability are required. Accordingly, this study investigated the novel role of MLT derivative, acetyl melatonin (ACT; Figure 1A), for its anti-CSC effect. The suppression of CSCs in human non-small cell lung cancer (NSCLC) by MLT and its derivative may imply their potential use as new anti-cancer agents in cancer treatment.

2 | RESULTS

2.1 | Effect of melatonin and acetyl melatonin on the viability of the human NSCLC cell line H460

Non-toxic concentrations of MLT and ACT were investigated to assess their effects on lung cancer stem cells. In cells treated with 1000 μmol/L MLT or 500 and 1000 μmol/L ACT, a significant decrease was observed in viability, while the non-toxic concentrations were 10–500 μmol/L MLT or 10–250 μmol/L ACT (Figure 1B). The assessment of cell death induction by acridine orange (AO)/propidium iodide (PI) staining confirmed the results of the cell viability tests. At the non-toxic concentrations of MLT (10–500 μmol/L) and ACT (10–250 μmol/L), cells did not show chromatin condensation and/or nuclear fragmentation, and, therefore, they were not apoptotic or necrotic (Figure 1C,D). The non-toxic concentrations of MLT and ACT were used for further experiments.

2.2 | Non-toxic concentrations of melatonin and acetyl melatonin suppress CSC and epithelial–mesenchymal transition phenotypes

The CSC-like phenotypes are commonly assessed through the capacity of cancer cell to form three-dimensional spheroids and proliferate in anchorage-independent cultivation.[12,28] The effects of MLT and ACT on NSCLC-derived H460 cell growth and survival were examined. The cells treated with non-toxic concentrations of MLT and ACT showed significant decreases in the number of colonies formed compared to untreated cells (Figure 2A). Spheroid growth was significantly inhibited in cells treated with MLT and ACT at 14 and 21 days of treatment (Figure 2B). These results indicate that MLT and ACT suppress the behaviours of CSCs in H460 cells. CSCs have been associated with the EMT of cells, thus increasing their metastatic ability. The effect of MLT and ACT on EMT was studied by determining cell migration behaviour. We observed that MLT and ACT treatment at non-cytotoxic concentrations (100 and 250 μmol/L) significantly decreased cell motility in the wound healing assay at 72 h of the treatment (Figure 2C).

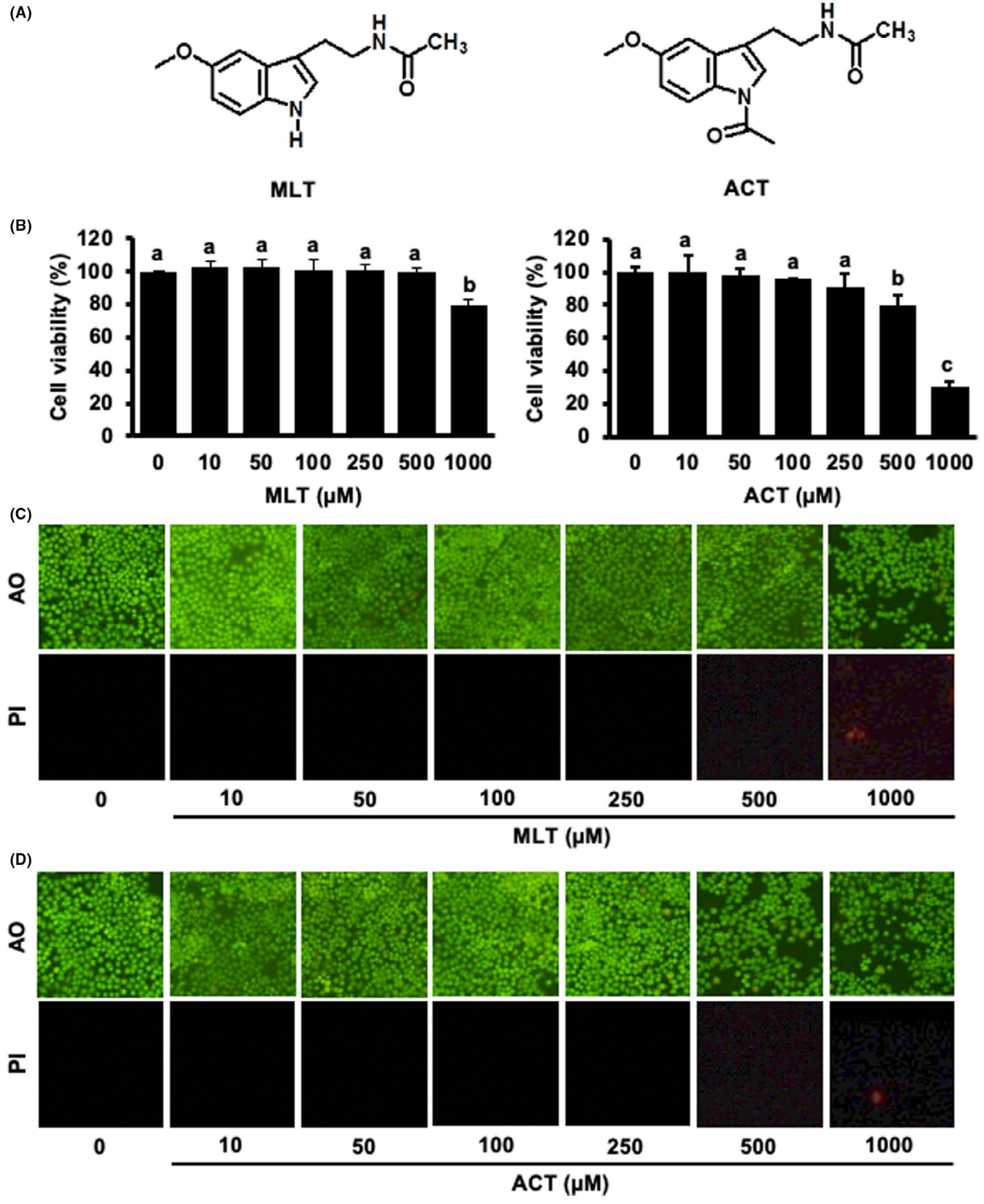


FIGURE 1 (A) Structure of melatonin (MLT) and acetyl melatonin (ACT). (B) Effect of MLT or ACT on cell viability. Human non-small cell lung cancer (NSCLC)-derived H460 cells were cultured with MLT or ACT for 24 h and subjected to be determined cell viability using MTT assay. Level of apoptotic and necrotic cell death was investigated by Acridine orange (AO)/propidium iodide (PI) co-staining after (C) MLT and (D) ACT treatment. Cells were imaged under fluorescence microscopy (4×). Data represent the mean ± SD ($n = 3$). Within each treatment group, the mean bars with same letters at the top of error bars indicate no significant difference

2.3 | Melatonin and acetyl melatonin decrease CSC and EMT markers

CD133 and ALDH1A1 are commonly used markers for identification of CSCs. Cells expressing CD133 and ALDH1A1 demonstrated an enhanced capacity for spheroid formation and chemoresistance.[5,6] We cultivated H460 cells for 48 h in the presence of MLT and ACT, and determined the levels of expression of CD133 and ALDH1A1 by western blotting. Results showed that the levels of expression of both markers were reduced by MLT and ACT (Figure 3). Moreover, MLT and ACT treatment resulted in lower levels of expression of EMT marker vimentin compared to the control (Figure 3). These findings indicate that MLT and ACT suppress CSC and EMT phenotypes in NSCLC-derived H460 cells.

2.4 | Melatonin and acetyl melatonin decrease self-renewal transcription factors

The self-renewal process divides stem cells into new identical stem cells through self-renewal transcription factors such as Oct-4 and Nanog.[7-9] These proteins are expressed in several types of CSC. In this study, H460 cells treated with MLT and ACT for 48 h had substantially reduced levels of Oct-4 and Nanog (Figure 4). This finding supports that MLT and ACT are negative regulators of CSCs by suppression the self-renewal transcription factors.

2.5 | Melatonin and acetyl melatonin suppress CSC phenotypes through an AKT-dependent mechanism that not mediated by membrane melatonin receptors

We studied the underlying mechanisms of action of MLT and ACT on CSC phenotypes in H640 cells. CSCs have previously been shown to increase and maintain their phenotype via the AKT signalling pathway through the protein β-catenin.[10-15] MLT and ACT treatment significantly decreased the expression of phosphorylated AKT (p-AKT) and β-catenin (Figure 4). These results indicate that MLT and ACT regulate CSCs by downregulation of AKT-dependent signalling.

MLT binds to MLT membrane receptors, MT1 and MT2.[32] We further evaluated the involvement of MLT receptors in the CSC inhibition pathway of MTL and ACT. Cells were pre-incubated with the MLT receptor antagonist luzindole (10 μmol/L) for 2 h, prior to 250 μmol/L MLT or ACT treatment. At 48 h of incubation, the expression of key molecular proteins p-AKT and AKT were determined. Pre-treatment of the cells with luzindole failed to prevent the attenuation of p-AKT. This indicates that CSC suppression by these compounds is independent of MT1 and MT2 receptors.

2.6 | Melatonin and acetyl melatonin inhibit enriched CSC population growth

The size of single spheroids decreased significantly with MLT and ACT at 5 and 7 days after treatment (Figure 5). At day 7 of treatment, spheroids were stained with PI, indicating that MLT and ACT exposure induced cell death. ACT was more cytotoxic than MLT to the enriched CSC population (Figure 5).

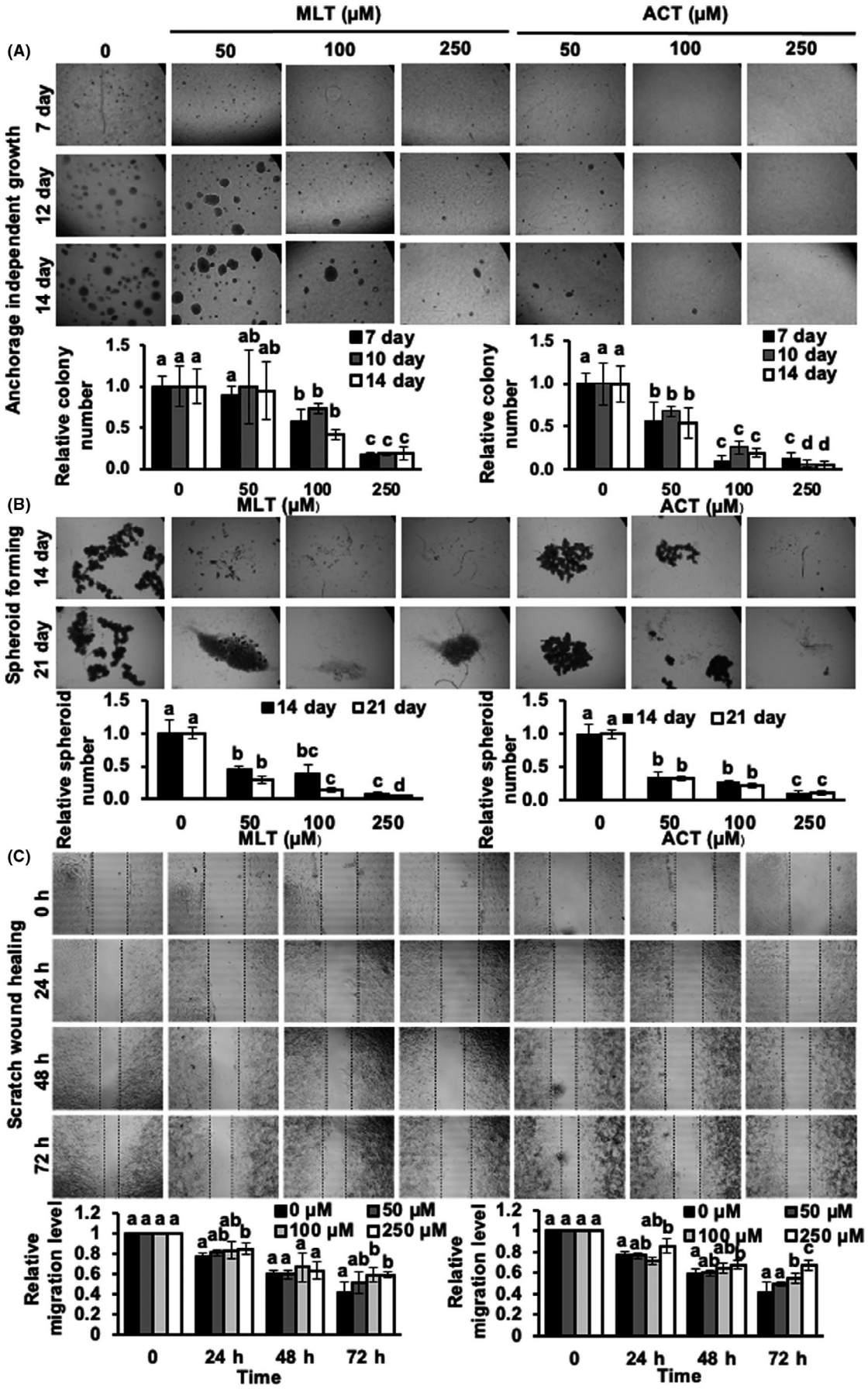
2.7 | Effects of melatonin and acetyl melatonin on CSCs from two other NSCLC-derived cell lines

After finding that MLT and ACT suppressed CSC phenotypes in NSCLC-derived H460 cells, their effects on other NSCLC-derived cell lines, H23 and A549, were also investigated. We observed that treatment with MLT and ACT decreased the number of colonies of both H23 and A549 cells (Figure 6A). In addition, spheroid size was reduced after MLT and ACT exposure (Figure 6B). MLT and ACT also inhibited cancer cell migration in both cell lines (Figure 6C). These data together confirmed our findings for H460 cells, showing that MLT and ACT suppressed CSC-like characteristics of human NSCLC cell lines.

3 | DISCUSSION

Lung cancer is now the most common cause of mortality from cancer in the world.[38,39] NSCLC is the major type of lung cancer, with a median progression time of about 5–24 months and frequent relapses in patients who have received chemotherapy.[40] Recent research has shown the major role of CSCs in cancer progression, metastasis and chemotherapy resistance;[1-4] therefore, successful new strategies to eliminate CSCs are required.[12,41] Recently, MLT was reported to suppress CSCs in a variety of cancers, such as breast, ovarian, brain, colon and lung.[27-31,42] The exact effect of MLT on lung CSCs and the underlying mechanisms of action are, however, largely unknown.

FIGURE 2 Effect of melatonin (MLT) and acetyl melatonin (ACT) on cancer stem cell (CSC) and epithelial-to-mesenchymal transition (EMT) behaviours in H460 cells. After MLT or ACT treatment for 48 h, cells were subject for (A) anchorage-independent growth assay, (B) spheroid forming assay, and (C) scratch wound healing assay. (A) The formation of colonies was recorded by microscopy (4×) at day 7, 12 and 14 of treatment and the relative colony number of MLT- or ACT-treated cells were compared to control group. Within the same day of treatment, the mean bars with same letters at the top of error bars indicate no significant difference. (B) Spheroid formation at day 14 and 21 was assessed by microscopy (4×). The relative colony number of MLT or ACT- treated cells were compared to control group. Within the same day of treatment, the mean bars with same letters at the top of error bars indicate no significant difference. (C) Phase-contrast images (4×) of migratory cells were captured at 0, 24, 48 and 72 h after treatment. The relative cell migration level at indicated time points was calculated relative to 0 h. Within the same time of treatment, the mean bars with same letters at the top of error bars indicate no significant difference


MLT (μM)
ACT (μM)
(A)
0
50
100
250
Anchorage independent growth
7 day
12 day
14 day
Relative colony number
7 day
10 day
14 day
(B)
Spheroid forming
14 day
21 day
Relative spheroid number
(C)
Scratch wound healing
0 h
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48 h
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Relative migration level
0 μM
50 μM
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Time

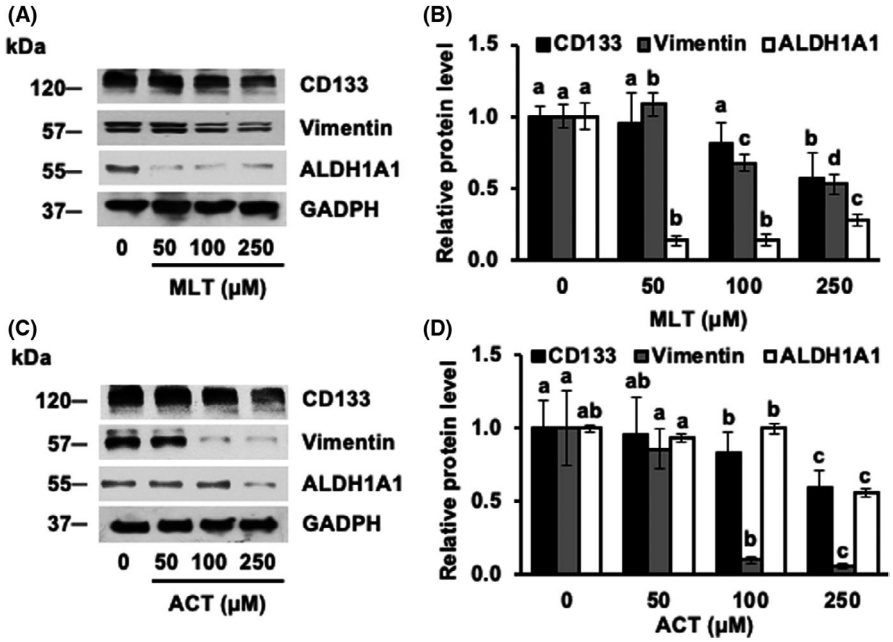


FIGURE 3 Melatonin (MLT) and acetyl melatonin (ACT) decreases the expression of cancer stem cell (CSC) and epithelial-to-mesenchymal transition (EMT) markers. H460 cells were treated with (A, B) MLT and (C, D) ACT for 48 h. Protein expression of CD133, vimentin and ALDH1A1 was examined by western blot analysis. Relative protein levels of treated H460 cells was compared to that of control group. Data represent mean ± SD ($n = 3$). Within each protein group, the mean bars with same letters at the top of error bars indicate no significant difference

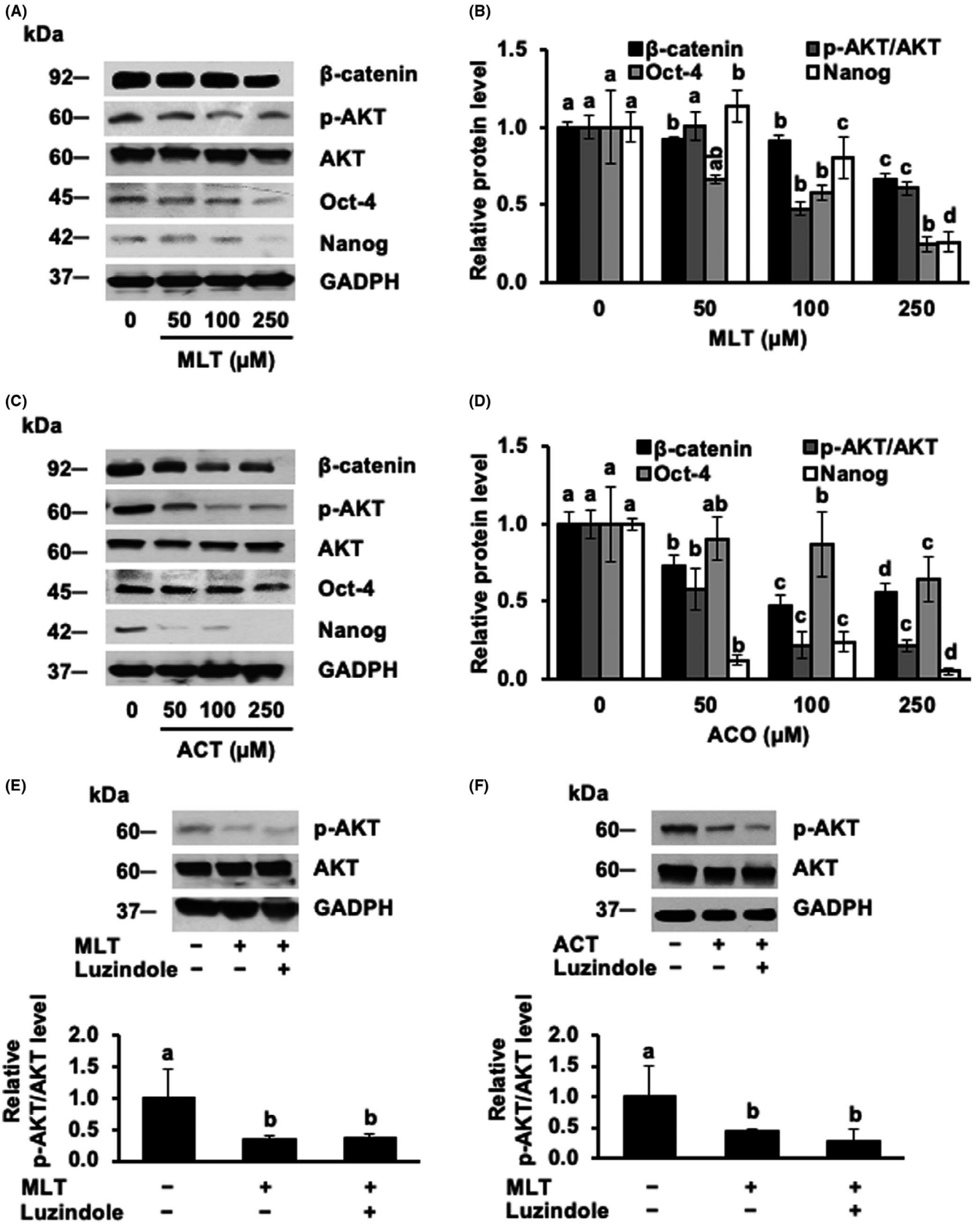
Our study provides an insight into MLT as a new agent for lung CSC suppression in three NSCLC-derived cell lines.

Our study found that non-toxic concentrations of MLT inhibited CSC-like phenotypes, and suppressed colony and spheroid formation in the H460, H23, and A549 cell lines (Figures 2, 5 and 6). The study by Yang et al. is the only other report of an effect of MLT on lung cancer stem cells. They found that 1 mM MLT reduced the formation of spheroids and expression of the stem cell marker CD133 in A549 and CL1-5 cell lines.[42] Our study provides further data on the effect of MLT against CSC markers, including reduced expression of both CD133 and ALDH1A1 caused by MLT exposure (Figure 3). AKT has previously been reported to be involved in CSC phenotype maintenance in various cancers, including lung cancer.[10-13] CSCs that expressed CD133 showed increased p-AKT expression.[43] Inhibition of AKT caused decreased Oct-4 levels and CSC proliferation.[44] Oct-4 is clearly phosphorylated by p-AKT, which also translocates into the nucleus to transcribe Nanog.[45] Our study found that MLT reduced the expression of p-AKT, leading to a decrease in the levels of Oct-4 and Nanog (Figure 4). Previous studies have shown the role of β-catenin in the aggressiveness and chemoresistance of lung cancers.[16,17] β-catenin is the upstream regulator of stemness, which increases Nanog and Oct-4 expression.[46] This study found down-expression of β-catenin after MLT treatment, confirming that MLT suppresses stemness through AKT/β-catenin reduction (Figure 4). In a previous study, CD133 may have been reduced by suppression of β-catenin, ERK/p38, PLC and Twist.[42]

Previous studies have reported that orally administered MLT has low bioavailability with wide variability between subjects.[36,37] To improve bioavailability, our group, therefore, synthesised new MLT derivatives, including two *N*-substituted derivatives—ACT and benzoyl melatonin. These derivatives were able to reduce the production of inflammatory mediators such as nitric oxide and prostaglandin E in macrophage cells and reduced pain in rats with prolonged action.[47] In this study, ACT suppressed CSC phenotypes by inhibiting colony and spheroid formation, and reduced the expression of CSC markers, CD133 and ALDH1A1 (Figures 2, 3, 5 and 6). The self-renewal markers, Oct-4 and Nanog, as well as upstream protein signalling, p-AKT, and β-catenin, were suppressed by ACT (Figure 4). Consistent with MLT, ACT suppressed stemness by mediating AKT/β-catenin signalling. Spheroid size and colony number were reduced more in the presence of ACT than of MLT (Figures 2a, 5, and 6a,b). The substitution of *N*-acetyl in MLT may improve lipophilicity, leading to increased permeability of ACT.[48,49]

The most functional cell membrane receptors of MLT are MT1 and MT2, which are in the G-protein coupled receptor family.[32] In uterine leiomyoma cells, MLT failed to reduce the expression of AKT when luzindole was added, indicating the role of the MLT receptors in AKT signalling suppression.[35] However, our recent study observed the independent of MLT receptors on the MLT-inhibited AKT signalling pathway. Furthermore, MLT acts through a receptor-independent manner, passing through the cell membrane and promoting radical scavenging properties.[50] The antioxidant effect of MLT was related to an anti-proliferative effect, through attenuation of AKT and NF-KB in C6 glioma cells.[51] In our study, the ability of MLT to change cellular redox state may inactivate the AKT signalling

FIGURE 4 Melatonin (MLT) and acetyl melatonin (ACT) inhibits the expression of self-renewal transcription factors and upstream signalling pathway. H460 cells were treated with (A, B) MLT and (C, D) ACT for 48 h. The expression level of β-catenin, p-AKT, AKT, Oct-4 and Nanog proteins were examined by western blot analysis. (E, F) Cotreatment of cells with (E) MLT or (F) ACT with the melatonin antagonist luzindole. Luzindole (10 μmol/L) was added 2 h before 250 μmol/L MLT or ACT treatment and then incubation of cells with both compounds for 48 h. The expression level of p-AKT and AKT proteins were determined by western blot analysis. Relative protein levels of treated H460 cells was compared to that of control group. Data represent mean ± SD ($n = 3$). Within each protein group, the mean bars with same letters at the top of error bars indicate no significant difference



pathway. Taken together, similar effects may be exhibited by ACT, since it has the antioxidant potential to regulate cellular redox states in cells.[52]

Studies increasingly show that CSCs possess EMT characteristics during lung cancer metastasis and EMT markers. EMT induction in cancer cells has been reported to increase stem cell-like

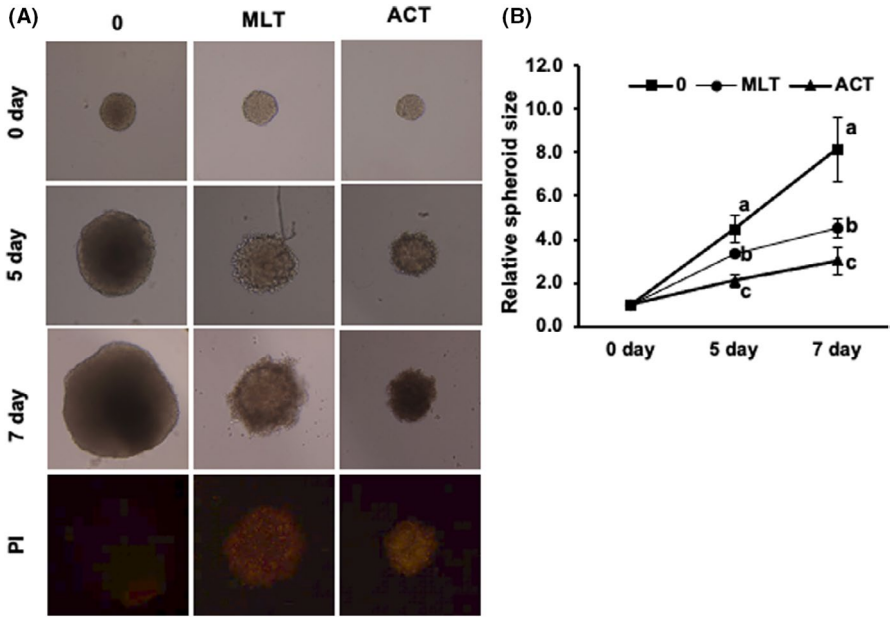


FIGURE 5 Melatonin (MLT) and acetyl melatonin (ACT) inhibits single cancer stem cell (CSC)-enriched spheroid growth. Single CSC-enriched spheroid derived from H460 cells were exposed to MLT (250 μmol/L) or ACT (250 μmol/L) for 7 days. (A) Spheroid images were captured by microscopy (10×) at day 0, 5, and 7 of treatment. Cell death was assessed by propidium iodide (PI) staining at day 7 of treatment. Cells were captured under fluorescence microscopy (10×). (B) The relative spheroid size at indicated time points was calculated relative to 0 h. Within the same time of treatment, the mean bars with same letters at the top of error bars indicate no significant difference

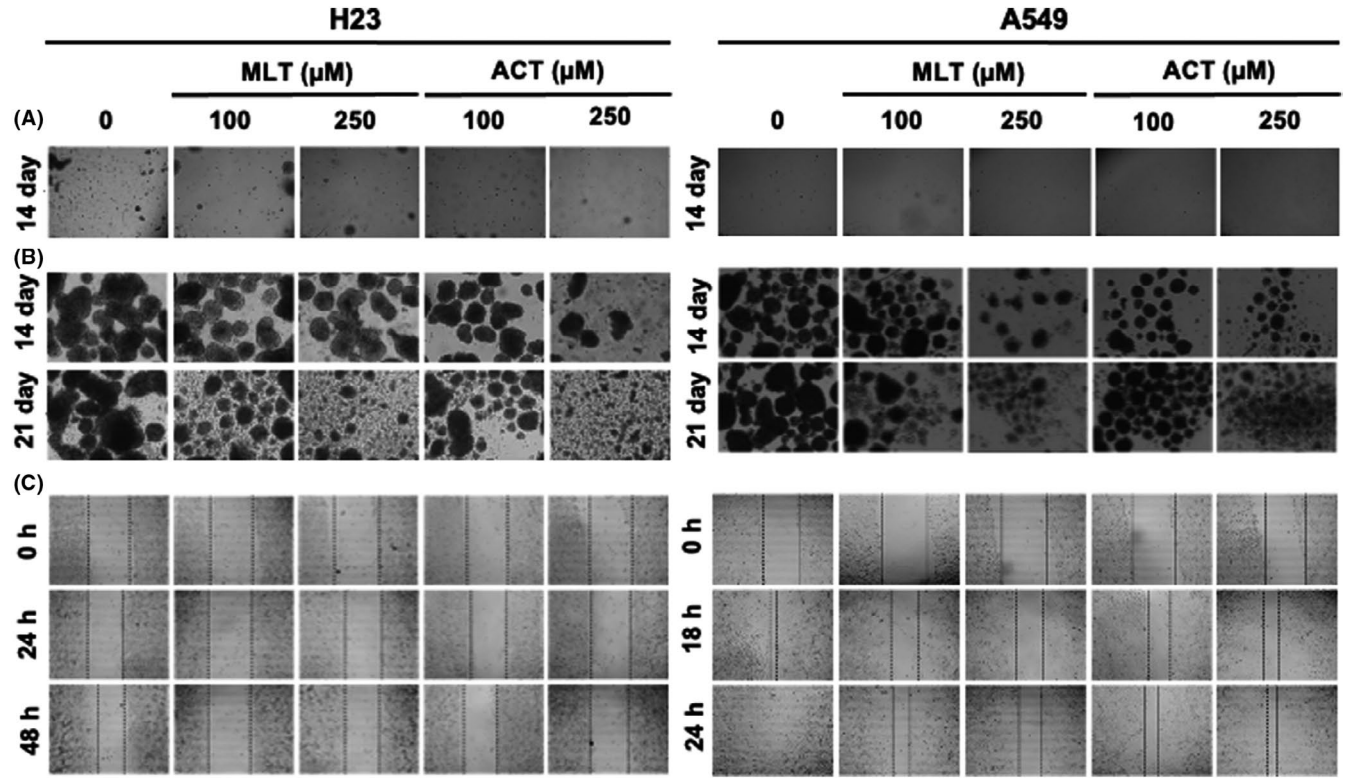


FIGURE 6 Melatonin (MLT) and acetyl melatonin (ACT) inhibits CSC-like behaviour in H23 and A549 cells. The respective cells were treated with MLT or ACT for 48 h. After treatment, cells were subject for (A) anchorage-independent growth assay, (B) spheroid forming assay, and (C) scratch wound healing assay. (A) The formation of colonies was recorded by microscopy (4×) at 14 of treatment. (B) Spheroid formation at day 14 and 21 was assessed by microscopy (4×). (C) Phase-contrast images (4×) of migratory cells were captured at 0, 24, and 48 for H23, and at 0, 18, and 24 h for A549 after treatment

phenotypes.[13,18,19] Strong upregulation of EMT markers vimentin and N-cadherin and EMT-related transcription factor Snail1 were found in CSC-NSCLC cells.[53] NSCLC patients with high vimentin-positive circulating tumour cell levels had a short progression-free survival after receiving epidermal growth factor receptor tyrosine kinase inhibitor therapy.[54] Our study found that MLT and ACT inhibited migration and downregulation of vimentin in NSCLC cells, suggesting that EMT regulation could suppress CSCs.

In conclusion, MLT and ACT are potentially beneficial anti-CSC agents, as they suppress CSC-like characteristics of NSCLC cell lines.

MLT and ACT reduce the level of active AKT, and inhibit the translocation of Oct-4 and β-catenin into the nucleus. Inhibition of key stemness proteins further suppresses Nanog, CSC markers (CD133, ALDH1A1), and the EMT marker (vimentin), as well as colony and spheroid formation (Figure 7). MLT is a safe ingredient commonly used in multivitamin and dietary supplement formulations. It is a promising candidate for further study in the treatment of lung cancer. However, the cellular mechanism of the anti-CSC effect should be further confirmed in the lung CSC model and *in-vivo* study. In the case of the new MLT-derivative ACT, its pharmacokinetic profiles as well as the MLT receptor binding affinity should be explored.

4 | MATERIALS AND METHODS

4.1 | Cell culture and reagents

Human NSCLC cell lines H460, H23, and A549 were bought from the American Type Culture Collection (Manassas, VA, USA). They were cultivated in Roswell Park Memorial Institute (RPMI) 1640 complete medium (RPMI 1640 with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 10% fetal bovine serum [FBS]). Cells were incubated under 5% CO_2 and 37°C humidity. Every two days, the cells were subcultured in a 0.25% trypsin solution. RPMI 1640 medium, penicillin/streptomycin, L-glutamine, FBS, and trypsin were bought from GIBCO. MLT was purchased from Shanghai Chemical Co. Ltd. The synthesis and characterisation of acetyl-melatonin (ACT; *N*-[2-(1-acetyl-5-methoxy-1H-indol-3-yl) ethyl]acetamide) was previously described in the Panyatip et al.[55] study. Luzindole (an antagonist of MT1 and MT2), AO and PI were purchased from Sigma Chemical. Bovine serum albumin, cell lysis buffer, and antibodies directed against CD133, vimentin, ALDH1A1, β-catenin, p-AKT, AKT, Oct-4, Nanog and GADPH, and secondary antibodies were bought from Cell Signaling Technology.

4.2 | Cell viability assay

Cell viability was examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. H460 cells (1×10^4 cells) were cultured overnight in 96-well plates. The cells were then exposed to MLT or ACT (0–1000 μmol/L) for 24 h. After that, the MLT and ACT solutions were discarded and MTT (400 μg/mL) was added and the cells incubated for 4 h under 5% CO_2 and 37°C humidity. The

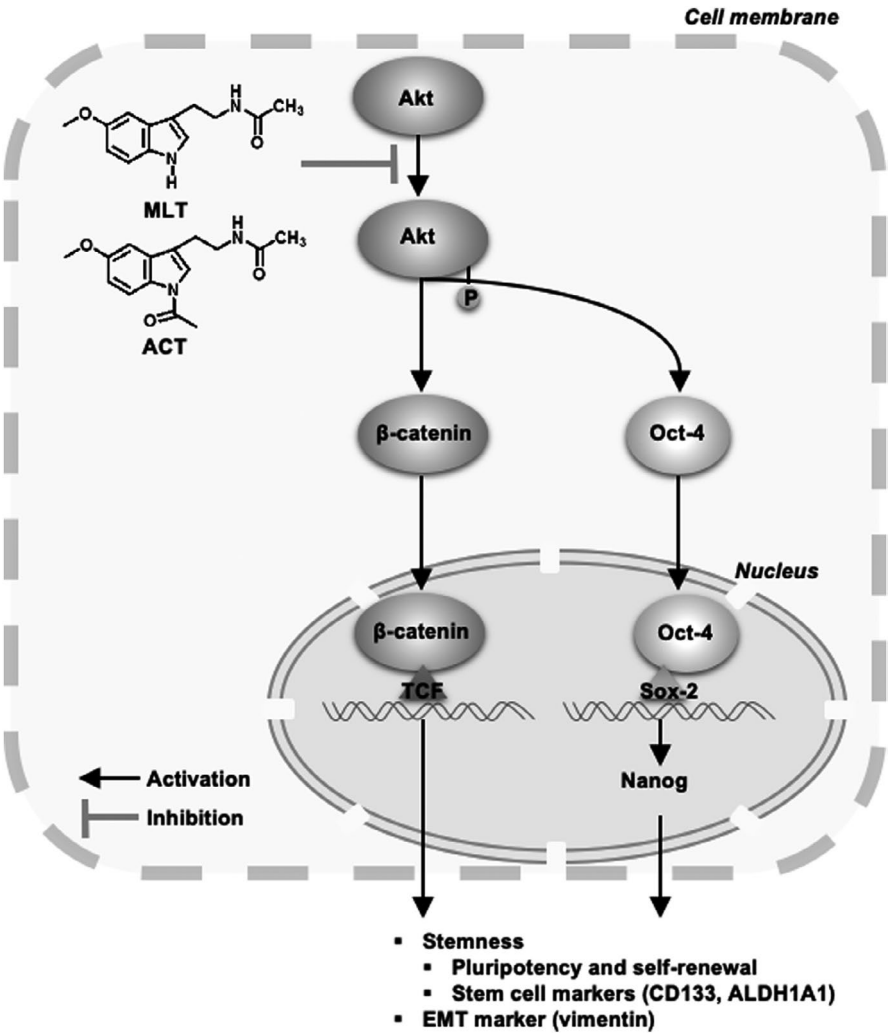


FIGURE 7 Schematic overview the cancer stem cell (CSC)-like phenotypes inhibition effect of melatonin (MLT) and acetyl melatonin (ACT) through AKT-dependent mechanism. MLT and ACT reduced the expression of phosphorylated AKT (p-AKT) and further downregulation of β-catenin and Oct-4. This resulted in the inhibition of CSC-like phenotypes in lung cancer cells

MTT solution was discarded, and replaced by 100 μL of dimethyl sulphoxide. The intensity of colour of formazan, formed by reduction of MTT, was determined by spectrophotometry at 570 nm (Bio-Rad Laboratories). The viability (%) of treated H460 cells was compared to that of control group.

4.3 | Cell death detection assay

H460 cells (1×10^4 cells) were cultured overnight in 96-well plates and exposed to MLT or ACT (0–1000 μmol/L). The compound solution was discarded after 24 h incubation. The cells were incubated in AO; 10 μg/mL) for 30 min and in PI; 5 μg/mL) for 5 min. Cells with DNA fragmentation and nuclear condensation, or PI-positive necrotic cells were captured under fluorescence microscopy (Olympus CKX41 inverted phase-contrast microscope with an Olympus E-330 camera; Olympus).

4.4 | Anchorage-independent growth assay

Proliferation of NSCLC H460, H23, and A549 cells was determined using a colony formation assay. Cells treated with MLT or ACT (50–250 μmol/L) for 48 h were cultured in soft agar for 14 days. Firstly, the lower layer was prepared using 1% (w/v) agarose in complete RPMI-1640 medium. Secondly, the cellular layer was prepared with cells (1×10^3) in 0.3% agarose in complete medium. Cells were then treated with MLT or ACT (50–250 μmol/L). Lastly, complete medium was added to the second layer. At days 7, 12, and 14, the colony was visualised under a phase-contrast microscope (Olympus CKX41 with an Olympus E-330 camera, Olympus).

4.5 | Three-dimensional spheroid formation assay

The NSCLC H460, H23, and A549 cells were pre-incubated with MLT or ACT (0–250 μmol/L) for 48 h. Cells (2.5×10^3 cells) were detached and cultured in a 24-well ultra-low attachment plate in RPMI 1640 medium contained 20 ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor (BD Biosciences), and 4 mg/mL insulin (Sigma). These cells were treated every two days for 21 days. Spheroids were visualised at day 14 and 21 using phase-contrast microscopy (Olympus IX51 with an Olympus DP70 camera, Olympus).

4.6 | Scratch wound healing assay

Cell migration were determined using a scratch wound healing assay. NSCLC H460, H23, and A549 cells were pre-incubated with MLT or ACT (0–250 μmol/L) for 48 h. Cells were detached and cultivated in a 96-well plate at 4×10^4 cells/well overnight. The cells attached were scratched with the tip of a pipette (20–200 μL). The old medium was replaced by a new complete medium with or without MLT or ACT, and further incubated for time points indicated below. Wound areas were imaged using bright field microscopy (10×) at 0, 24, 48, and 72 h for H460; at 0, 24, and 48 for H23 cells; and at 0, 18, and 24 h for A549 cells. The width of the wound areas was measured by ImageJ software. Cell migration at indicated time points was calculated relative to 0 h.

4.7 | Western blot analysis

H460 cells (1×10^5 cells) were treated with 50–250 μmol/L MLT or ACT for 48 h. For luzindole cotreatment experiments, cells were pretreated with luzindole (10 μmol/L) for 2 h and then further incubation with 250 μmol/L MLT or ACT for 48 h. Cells were lysed using cell lysis buffer (Cell Signaling Technology) contained protease inhibitor cocktail (Bio-Rad), 100 mmol/L phenylmethylsulfonyl fluoride, and 0.5% (v/v) Triton X-100. The level of protein of cell lysates was analysed by protein assay kit (Bio-Rad). Each cell lysate was loaded to a sodium dodecyl sulphate polyacrylamide gel. The separated proteins were moved to membranes (0.45 μm nitrocellulose) and block with 5% (w/v) non-fat milk in TBST solution for 45 mins. TBST solution contained 125 mmol/L NaCl, 25 mmol/L Tris-HCl, and 0.1% [v/v] Tween 20. The blots were exposed to primary antibodies (CD133, vimentin, ALDH1A1, β-catenin, p-AKT, AKT, Oct-4, Nanog and GADPH) overnight at 4°C. The membranes were washed with TBST and further incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h. The level of each protein was determined by chemiluminescence substrate (Bio-Rad). The intensity of protein band was measured using ImageJ software.

4.8 | Single spheroid formation assay

The spheroids were formed as described above in the spheroid formation assay for 14 days. Each individual spheroid was separated and cultured in a 96-well ultra-low attachment well plate and incubated with 250 μmol/L MLT or ACT every two days for 7 days. Spheroids were visualised using phase-contrast microscopy (Olympus IX51 with Olympus DP70 camera, Olympus) at day 5 and 7. Relative spheroid size was calculated for the treatments and compared to that of the untreated control.

4.9 | Statistical analysis

Means ±standard deviation (SD) of the data in each experiment are presented. Multiple comparisons were analysed with analysis of variance (ANOVA), and post hoc multiple means comparisons performed with Scheffé's test at the $p < .05$ significance level.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in regard to this study.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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REFERENCES

1. Miyoshi N, Mizushima T, Doki Y, Mori M. Cancer stem cells in relation to treatment. *Jpn J Clin Oncol*. 2019;49(3):232-237.
2. Vega FM, Colmenero-Repiso A, Gómez-Muñoz MA, et al. CD44-high neural crest stem-like cells are associated with tumour aggressiveness and poor survival in neuroblastoma tumours. *EBioMedicine*. 2019;49:82-95.
3. Toledo-Guzmán ME, Bigoni-Ordóñez GD, Ibáñez Hernández M, Ortiz-Sánchez E. Cancer stem cell impact on clinical oncology. *World J Stem Cells*. 2018;10(12):183-195.
4. Sen HW, Gosens R, Kruyt FAE. Lung cancer stem cells: origin, features, maintenance mechanisms and therapeutic targeting. *Biochem Pharmacol*. 2019;160:121-133.
5. Alamgeer M, Ganju V, Szczepny A, et al. The prognostic significance of aldehyde dehydrogenase 1A1 (ALDH1A1) and CD133 expression in early stage non-small cell lung cancer. *Thorax*. 2013;68(12):1095-1104.
6. Miyata T, Oyama T, Yoshimatsu T, et al. The clinical significance of cancer stem cell markers ALDH1A1 and CD133 in lung adenocarcinoma. *Anticancer Res*. 2017;37(5):2541-2547.
7. Wahab SMR, Islam F, Gopalan V, Lam AK-Y. The identifications and clinical implications of cancer stem cells in colorectal cancer. *Clin Colorectal Cancer*. 2017;16(2):93-102.
8. Sławek S, Szmyt K, Fularz M, et al. Pluripotency transcription factors in lung cancer – a review. *Tumour Biol J Int Soc Oncodevelopmental Biol Med*. 2016;37(4):4241-4249.
9. Chiou S-H, Yu C-C, Huang C-Y, et al. Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. *Clin cancer Res an Off J Am Assoc Cancer Res*. 2008;14(13):4085-4095.
10. Hou Y, Li H, Huo W. THBS4 silencing regulates the cancer stem cell-like properties in prostate cancer via blocking the PI3K/Akt pathway. *Prostate*. 2020;80(10):753-763.
11. Jabbarzadeh Kaboli P, Salimian F, Aghapour S, et al. Akt-targeted therapy as a promising strategy to overcome drug resistance in breast cancer – a comprehensive review from chemotherapy to immunotherapy. *Pharmacol Res*. 2020;156:104806.
12. Phiboonchaiyanan PP, Chanvorachote P. Suppression of a cancer stem-like phenotype mediated by alpha-lipoic acid in human lung cancer cells through down-regulation of beta-catenin and Oct-4. *Cell Oncol (Dordr)*. 2017;40(5):497-510.
13. Wang H, Zhang G, Zhang H, et al. Acquisition of epithelial-mesenchymal transition phenotype and cancer stem cell-like properties in cisplatin-resistant lung cancer cells through AKT/β-catenin/Snail signaling pathway. *Eur J Pharmacol*. 2014;723:156-166.
14. Duda P, Akula SM, Abrams SL, et al. Targeting GSK3 and associated signaling pathways involved in cancer. *Cells*. 2020;9(5):1110.
15. Verheyen EM, Gottardi CJ. Regulation of Wnt/beta-catenin signaling by protein kinases. *Dev Dyn*. 2010;239(1):34-44.
16. Teng Y, Wang X, Wang Y, Ma D. Wnt/beta-catenin signaling regulates cancer stem cells in lung cancer A549 cells. *Biochem Biophys Res Commun*. 2010;392(3):373-379.
17. Mohammed MK, Shao C, Wang J, et al. Wnt/β-catenin signaling plays an ever-expanding role in stem cell self-renewal, tumorigenesis and cancer chemoresistance. *Genes Dis*. 2016;3(1):11-40.
18. Mani SA, Guo W, Liao M-J, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008;133(4):704-715.
19. Strouhalova K, Přechová M, Gandalovičová A, Brábek J, Gregor M, Rosel D. Vimentin intermediate filaments as potential target for cancer treatment. *Cancers (Basel)*. 2020;12(1):184.
20. Zhao D, Yu Y, Shen Y, et al. melatonin synthesis and function: evolutionary history in animals and plants. *Front Endocrinol (Lausanne)*. 2019;10:249.
21. Morera-Fumero AL, Fernandez-Lopez L, Abreu-Gonzalez P. Melatonin and melatonin agonists as treatments for benzodiazepines and hypnotics withdrawal in patients with primary insomnia. A systematic review. *Drug Alcohol Depend*. 2020;212:107994.
22. Liampas I, Siokas V, Brotis A, Vikelis M, Dardiotis E. Endogenous melatonin levels and therapeutic use of exogenous melatonin in migraine: systematic review and meta-analysis. *Headache*. 2020;60(7):1273-1299.
23. Tamtaji OR, Reiter RJ, Alipoor R, Dadgostar E, Kouchaki E, Asemi Z. Melatonin and parkinson disease: current status and future perspectives for molecular mechanisms. *Cell Mol Neurobiol*. 2020;40(1):15-23.
24. Pourhanifeh MH, Sharifi M, Reiter RJ, Davoodabadi A, Asemi Z. Melatonin and non-small cell lung cancer: new insights into signaling pathways. *Cancer Cell Int*. 2019;19:131.
25. Gil-Martín E, Egea J, Reiter RJ, Romero A. The emergence of melatonin in oncology: focus on colorectal cancer. *Med Res Rev*. 2019;39(6):2239-2285.
26. Ma N, Zhang J, Reiter RJ, Ma X. Melatonin mediates mucosal immune cells, microbial metabolism, and rhythm crosstalk: a therapeutic target to reduce intestinal inflammation. *Med Res Rev*. 2020;40(2):606-632.
27. Akbarzadeh M, Rahbarghazi R, Nabat E, et al. The impact of different extracellular matrices on melatonin effect in proliferation and stemness properties of ovarian cancer cells. *Biomed Pharmacother*. 2017;87:288-295.
28. Gonçalves NdoN, Colombo J, Lopes JR, et al. Effect of melatonin in epithelial mesenchymal transition markers and invasive properties of breast cancer stem cells of canine and human cell lines. *PLoS One*. 2016;11(3):e0150407.
29. Lopes JR, da Silva KM, de Medeiros FAF, de Campos Zuccari DAP. Evaluation of melatonin effect on human breast cancer stem cells using a threedimensional growth method of mammospheres. *Anticancer Agents Med Chem*. 2017;17(7):961-965.
30. Martín V, Sanchez-Sanchez AM, Herrera F, et al. Melatonin-induced methylation of the ABCG2/BCRP promoter as a novel mechanism to overcome multidrug resistance in brain tumour stem cells. *Br J Cancer*. 2013;108(10):2005-2012.
31. Chen X, Hao A, Li X, et al. Melatonin inhibits tumorigenicity of glioblastoma stem-like cells via the AKT-EZH2-STAT3 signaling axis. *J Pineal Res*. 2016;61(2):208-217.

32. Liu J, Clough SJ, Hutchinson AJ, Adamah-Biassi EB, Popovska-Gorevski M, Dubocovich ML. MT1 and MT2 melatonin receptors: a therapeutic perspective. *Annu Rev Pharmacol Toxicol*. 2016;56:361-383.
33. Mao L, Cheng Q, Guardiola-Lemaître B, et al. In vitro and in vivo antitumor activity of melatonin receptor agonists. *J Pineal Res*. 2010;49(3):210-221.
34. Chao C-C, Chen P-C, Chiou P-C, et al. Melatonin suppresses lung cancer metastasis by inhibition of epithelial-mesenchymal transition through targeting to Twist. *Clin Sci (Lond)*. 2019;133(5):709-722.
35. Lin P-H, Tung Y-T, Chen H-Y, et al. Melatonin activates cell death programs for the suppression of uterine leiomyoma cell proliferation. *J Pineal Res*. 2020;68(1):e12620.
36. DeMuro RL, Nafziger AN, Blask DE, Menhinick AM, Bertino JSJ. The absolute bioavailability of oral melatonin. *J Clin Pharmacol*. 2000;40(7):781-784.
37. Claustrat B, Brun J, Chazot G. The basic physiology and pathophysiology of melatonin. *Sleep Med Rev*. 2005;9(1):11-24.
38. Ferlay J, Colombet M, Soerjomataram I, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J cancer*. 2019;144(8):1941-1953.
39. Sharma D, Newman TG, Aronow WS. Lung cancer screening: history, current perspectives, and future directions. *Arch Med Sci*. 2015;11(5):1033-1043.
40. Ashworth A, Rodrigues G, Boldt G, Palma D. Is there an oligometastatic state in non-small cell lung cancer? A systematic review of the literature. *Lung Cancer*. 2013;82(2):197-203.
41. Shibata M, Hoque MO. Targeting cancer stem cells: a strategy for effective eradication of cancer. *Cancers*. 2019;11(5):732.
42. Yang Y-C, Chiou P-C, Chen P-C, et al. Melatonin reduces lung cancer stemness through inhibiting of PLC, ERK, p38, beta-catenin, and Twist pathways. *Environ Toxicol*. 2019;34(2):203-209.
43. Wei Y, Jiang Y, Zou F, et al. Activation of PI3K/Akt pathway by CD133-p85 interaction promotes tumorigenic capacity of glioma stem cells. *Proc Natl Acad Sci U S A*. 2013;110(17):6829-6834.
44. Zhao Q-W, Zhou Y-W, Li W-X, et al. Akt-mediated phosphorylation of Oct4 is associated with the proliferation of stem-like cancer cells. *Oncol Rep*. 2015;33(4):1621-1629.
45. Lin Y, Yang Y, Li W, et al. Reciprocal regulation of Akt and Oct4 promotes the self-renewal and survival of embryonal carcinoma cells. *Mol Cell*. 2012;48(4):627-640.
46. Jiang R, Niu X, Huang Y, Wang X. β-Catenin is important for cancer stem cell generation and tumorigenic activity in nasopharyngeal carcinoma. *Acta Biochim Biophys Sin (Shanghai)*. 2016;48(3):229-237.
47. Phiphatwatcharaded C, Topark-Ngarm A, Puthongking P, Mahakunakorn P. Anti-inflammatory activities of melatonin derivatives in lipopolysaccharide-stimulated RAW 264.7 cells and antinociceptive effects in mice. *Drug Dev Res*. 2014;75(4):235-245.
48. Tabba HD, Abdel-Hamid ME, Suleiman MS, et al. Synthesis, identification and preliminary evaluation of esters and amide derivatives of diflunisal. *Int J Pharm*. 1989;54(1):57-63.
49. Simões MF, Valente E, Gómez MJR, Anes E, Constantino L. Lipophilic pyrazinoic acid amide and ester prodrugs stability, activation and activity against *M. tuberculosis*. *Eur J Pharm Sci Off J Eur Fed Pharm Sci*. 2009;37(3–4):257-263.
50. Witt-Enderby PA, Radio NM, Doctor JS, Davis VL. Therapeutic treatments potentially mediated by melatonin receptors: potential clinical uses in the prevention of osteoporosis, cancer and as an adjuvant therapy. *J Pineal Res*. 2006;41(4):297-305.
51. Martín V, Herrera F, Carrera-Gonzalez P, et al. Intracellular signaling pathways involved in the cell growth inhibition of glioma cells by melatonin. *Cancer Res*. 2006;66(2):1081-1088.
52. Phiphatwatcharaded C, Puthongking P, Chaiyarit P, Johns NP, Sakolchai S, Mahakunakorn P. The anti-oxidant effects of melatonin derivatives on human gingival fibroblasts. *Arch Oral Biol*. 2017;79:55-61.
53. Gomez-Casal R, Bhattacharya C, Ganesh N, et al. Non-small cell lung cancer cells survived ionizing radiation treatment display cancer stem cell and epithelial-mesenchymal transition phenotypes. *Mol Cancer*. 2013;12(1):94.
54. Hsu C-L, Tsai T-H, Huang C-K, et al. Monitoring levels of vimentin-positive circulating cancer stem cells and tumor cells in patients with advanced EGFR-mutated non-small cell lung cancer. *Lung Cancer*. 2021;156:50-58.
55. Panyatip P, Pratheepawanit Johns N, Priprem A, Nakagawa K, Puthongking P. Effect of N-amide substitution on antioxidative activities of melatonin derivatives. *Sci Pharm*. 2020;88(1):3.

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