Original Article

A Novel Combination of Melittin and Palbociclib for Esophageal Cancer: In Vitro Evidence of Synergy and Mechanistic Insights

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ABSTRACT

Objective: To measured the possible synergistic effect of melittin in combination with palbociclib against KYSE-30 esophageal squamous cell carcinoma cells.
Study Design: A inivtro study.
Place and Duration of Study: This study was conducted at the College of Medicine, University of Babylon, Iraq from 1st June 2025 to 30th November 2025.
Methods: KYSE-30 and HEK normal cells were treated with melittin (0.15625-20 µg/mL) and palbociclib (0.625-80 µg/mL) and then KYSE-30 were treated with combination of these two substances. Using the MTT test, we calculated the cell viability. The cyclin-D1, Nrf2 and IL-6 concentrations using enzyme-linked immunosorbent assay. Cell migration was assessed by wound healing assay.
Results: Melittin (5-10 µg/mL) significantly reduced KYSE-30 viability ($p < 0.05$) but only affected HEK cells at 20 µg/mL ($p<0.001$). Palbociclib (20-80 µg/mL) reduced KYSE-30 viability with an IC_{50} of 22 µg/mL. Palbociclib alone significantly increased cyclin-D1 ($P<0.001$), while the combination of melittin (10 µg/mL) + palbociclib (22 µg/mL) significantly decreased cyclin-D1 ($P=0.048$). Palbociclib (11 µg/mL) increased Nrf2 ($P=0.029$), but the combination decreased Nrf2 ($P<0.05$).When compared to palbociclib alone, the combination approach produced greater suppression of IL-6 levels ($P=0.016$, $P=0.004$) and inhibited cell migration with wound expansion at higher doses.
Conclusion: In KYSE-30 cells, the combination treatment with melittin (10 µg/mL) and palbociclib (22 µg/mL) produced a pharmacodynamic potentiation on cell viability, regulation of cell cycle, antioxidant response, inflammation, and migration, suggesting the need for additional in vivo studies.
Key Words: Esophageal squamous cell carcinoma, Melittin, Palbociclib, Combination therapy, Cyclin-D1

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INTRODUCTION

Esophageal cancer represents one of the most lethal and aggressive tumor types worldwide.[1,4] Its ranking among cancers globally is eleventh, and among cancer mortality, it ranks seventh.[4,5] It has two histological types: esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (EAC).[1,4] ESCC is most common in East and Middle Asia, especially in developing countries, linked to smoking and diet.[4,6] EAC is common in Western countries, linked to GERD and Barrett's esophagus.[2,4] ESCC represents 85% of esophageal cancers.[4] Its rapid progression and late diagnosis make it difficult to treat.[7,8]

In spite of a strong biological rationale for targeting the Cyclin D-CDK4/6-RB pathway in ESCC, no CDK4/6 inhibitor is currently approved for this disease.[4,9] Palbociclib, a selective CDK4/6 inhibitor, induces arrest of cell cycle at G1 phase but produces primarily a cytostatic effect rather than direct cancer cell death.[9,10] The mechanisms help ESCC cells survive treatment. These mechanisms develop quickly and activation of the PI3K/AKT/mTOR pathway. They also include ERBB/RTK signaling. Cancer cells can change their metabolism. They can use autophagy. They also avoid being recognized by the body's immune system.[11,12] Thus, treatment with palbociclib alone does not work for ESCC patients. This necessitates proper combination therapies that inhibit cell cycle progress and avoid other survival mechanisms.[9,13] Melittin is a natural compound that comes from bee venom. It works as an anticancer agent through cell membrane disruption.[14,15]

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This, however, requires large amounts of melittin that have toxic effects on normal cells. Palbociclib arrests ESCC cell growth. This arrest may sensitize cells to melittin.[16] Thus, a low-dose melittin combination with palbociclib may be effective and less toxic.

METHODS

This inivtro study was conducted at College of Medicine, University of Babylon, Iraq from 1st June 2025 to 30th November 2025 vide letter No. 152/QM/Approval/KDJEGVVD dated May 17, 2025. We obtained KYSE-30 esophageal cancer cells and HEK normal cells from the Tissue Culture Laboratory at the College of Medicine, University of Babylon. These cell lines were propagated in RPMI-1640 medium (Gibco, UK) fortified with 10% fetal bovine serum (FBS, Gibco, UK), 1% penicillin/streptomycin, and 50 µg/mL gentamicin (Ajanta, India), and incubated at 37°C under 5% CO_2 atmosphere. In distilled water Melittin (Shyuanye, China) was dissolved to prepare a stock solution of 1 mg/mL. Palbociclib (Bidepharm, China) was dissolved in dimethyl sulfoxide (DMSO, Roth, Germany) to produce a stock solution of 1 mg/mL. Working concentrations were prepared by dilution in culture medium immediately before each experiment.

Phosphate-buffered saline (PBS) was prepared by dissolving one tablet (Gibco, UK) in deionized distilled water and sterilized by autoclaving at 120°C for 20 minutes. Trypsin-EDTA solution was prepared by dissolving trypsin-EDTA powder (Gibco, UK) in deionized distilled water, adjusting the pH to 7.2, and sterilizing by serial filtration through 0.45 µm and 0.22 µm filters (Biofil, China). MTT solution (5 mg/mL) was prepared by dissolve MTT powder (Roth, Germany) in PBS, sterilized using a 0.22 µm filter (Biofil, China), and stored in a light-protected bottle at 4°C.

Cell viability was assessed using the MTT assay. A total of $2 \times 10_4$ cells were placed into each well of 96-well plates and allowed to attach overnight at 37°C. Following drug treatment, MTT working solution (0.5 mg/mL) was introduced and plates were incubated for an additional 4 hours at 37°C.[19,20] The culture medium was subsequently removed, and 100 µL of DMSO was added to solubilize the formazan precipitate. Optical density was determined at 570 nm with a microplate reader (Human, Germany). Cell viability was calculated using the formula:

Cell viability (%) = (Abs sample - Abs blank) / (Abs control - Abs blank) × 100

Melittin was tested on KYSE-30 cells at concentrations of 0.3125, 0.625, 1.25, 2.5, 5, and 10 µg/mL for 24 hours. On HEK normal cells, melittin was tested at concentrations of 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 µg/mL for 24 hours. Palbociclib was tested on both KYSE-30 and HEK cells at concentrations of 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 µg/mL for 48 hours.

The IC_{50} was calculated by generating a standard curve of cell inhibition percentage versus log drug concentration using Microsoft Excel. The concentration equivalent to 50% inhibition was calculated from the dose-response curve.

We tested the combination on KYSE-30 cells. The treatment lasted 48 hours. We used a fixed dose of palbociclib (22 µg/mL). We added different doses of melittin. These were 0.625, 1.25, 2.5, 5, 10, and 20 µg/mL. Then we measured cell survival. We did this using the MTT test.[19,20]

Following 48 hours of incubation, the cell culture supernatant was harvested for the determination of IL-6 levels. In case of intracellular markers such as Cyclin-D1 and Nrf2, cells were lysed using RIPA buffer. ELISA sandwich assays for all markers were conducted according to the manufacturer's guidelines using kits from Sunlong (China). The absorbance reading was carried out at 450 nm wavelength using a Human ELISA reader from Germany.

Cell culture of KYSE-30 cells was performed on 48-well plates (Biofil, China) till they became 90-100% confluent. At this point, a scratch line was created in all wells using sterile tips of a 200 µL pipette. The cell monolayers were gently washed with PBS to create clear areas, as per standardized procedures.[22,23] Next, we added drugs to the wells. Some wells got melittin (2.5 or 5 µg/mL). Some got palbociclib (2.5 or 5 µg/mL). Some got both (2.5+2.5, 2.5+5, or 5+5 µg/mL). We mixed all drugs in serum-free medium. We also had control wells. One control had no drugs. Another had only the vehicle. The pictures of the scratches at 0, 24, and 48 hours were taken. We used an inverted microscope from T.C Meiji Tech (Japan). We measured cell movement using Image J software.

We conducted all experiments in triplicate and expressed the data as mean ± standard deviation (SD). Using Sigma Plot, we performed one-way ANOVA with Holm-Sidak correction (MTT assay) or Tukey's post-hoc test (scratch assay) to determine statistical significance. We considered $p \leq 0.05$ to be statistically significant.

RESULTS

Melittin treatment inhibited the growth of KYSE-30 and HEK cells in a concentration-dependent manner. Melittin at 5 and 10 µg/mL significantly reduced KYSE-30 cell viability compared to control ($p<0.001$). Concentrations of 0.3125-2.5 µg/mL showed no significant effect ($p>0.05$) [Fig. 1A]. Melittin significantly reduced HEK cell viability only at the highest concentration tested (20 µg/mL) ($p<0.001$). No significant effect was observed at 0.15625-10 µg/mL ($p>0.05$) [Fig. 1B].

Palbociclib at 20, 40, and 80 µg/mL significantly reduced KYSE-30 viability ($p<0.001$). Lower concentrations (0.625-10 µg/mL) had no effect. The IC_{50} was calculated to be 22 µg/mL [Fig. 2A]. Palbociclib at 20, 40, and 80 µg/mL also significantly reduced HEK viability ($p<0.05$), while concentrations of 2.5-10 µg/mL showed no significant effect ($p > 0.05$) [Fig. 2B].

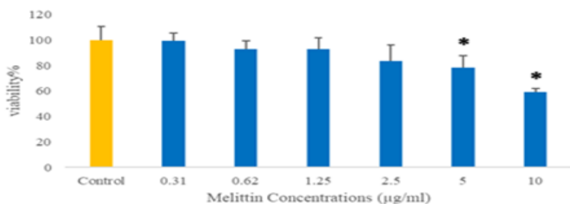
Fixed palbociclib (22 µg/mL) combined with melittin (0.625-20 µg/mL) significantly reduced KYSE-30 viability at all concentrations compared to control ($P<0.05$). Compared to palbociclib alone, combinations with melittin at 5, 10, and 20 µg/mL showed significantly greater cytotoxicity ($P<0.001$). Lower melittin doses (0.625, 1.25, and 2.5 µg/mL) did not significantly differ from palbociclib alone ($P>0.05$) [Fig. 3]/

Palbociclib at 11 µg/mL did not significantly alter Cyclin-D1 levels in KYSE-30 cells compared to control (Dunn's method, $P>0.05$) while Palbociclib at 22 µg/mL significantly increase Cyclin-D1 level. A non-significant reduction was observed at 11 µg/mL (2.0 vs. 2.6 pg/mL) (Fig. 4A). Melittin at 11 µg/mL and 22 µg/mL did not significantly alter Cyclin-D1 levels (Dunn's method, $P>0.05$) (Fig. 4B). In comparison to Palbociclib, the combination of melittin 10, 12.5 and 20 µg/mL + palbociclib 22 µg/mL significantly decreased Cyclin-D1 levels the combination of (melittin 2.5 µg/mL with palbociclib) did not show significant changes ($P > 0.05$) (Fig. 4C).

Palbociclib at 11 µg/mL significantly increased Nrf2 levels compared to control ($P = 0.028$). Palbociclib at 22 µg/mL did not significantly alter Nrf2 levels ($P=0.127$) (Fig. 5A). Melittin significantly reduced Nrf2 levels at both 6.25 µg/mL ($P=0.024$) and 12.5 µg/mL ($P=0.013$) compared to control (Fig. 5B). The combination of melittin 10 and 12.5 µg/mL + palbociclib 22 µg/mL significantly decreased Nrf2 levels compared to control ($P<0.05$). Other combinations (melittin 12.5, and 20 µg/mL with palbociclib) and palbociclib alone did not show significant changes ($P > 0.05$) while the combination 2.5 µg/mL melittin and 22 palbociclib is significantly increase Nrf2 level ($P=0.016$) (Fig. 5C)

Melittin at 6.25 and 12.5 µg/mL did not significantly alter IL-6 levels ($P=0.257$) [Fig. 6A]. Palbociclib also at 11 and 22 µg/mL did not alter IL-6 levels ($P=0.089$) (Fig. 6B). Palbociclib alone (22 µg/mL) did not significantly alter IL-6 levels compared to control ($P=0.080$). However, the combinations of melittin 10 and 12 µg/mL + palbociclib 22 µg/mL ($P = 0.016$) and melittin 12.5 µg/mL + palbociclib 22 µg/ml ($P=0.004$) significantly decreased IL-6 levels compared to palbociclib alone. No significant differences were observed compared to the control group (Fig. 6C).

The wound healing assay was accomplished to estimate cell migration. The control group achieved complete wound closure at 48 hours (from 330 pixels at 0 h to 0 pixels at 48 h), confirming normal cell migration. Treatment with palbociclib alone at 2.5 and 5 µg/mL significantly inhibited cell migration, with wound areas remaining high at 48 hours (324 pixels for both concentrations), corresponding to wound closure percentages of 3.8% and 1.8%, respectively. The combination of melittin 2.5 µg/mL + palbociclib 2.5 µg/mL showed similar inhibition (1.4% closure), with no significant difference compared to palbociclib alone. However, the combinations meliin 2.5 µg/mL + palbociclib 5 µg/mL and melittin 5 µg/mL + palbociclib 5 µg/mL resulted in wound expansion, with closure percentages of -2.7% and -3.2%, respectively, indicating a cytotoxic effect at higher combination doses (Table 1).



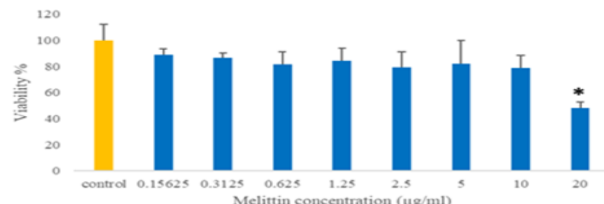
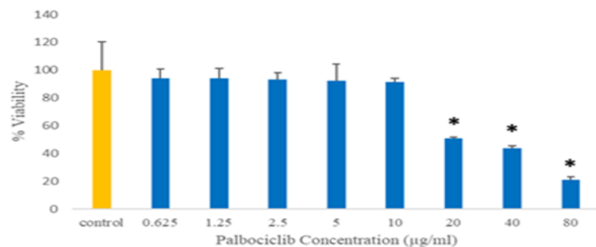


Figure No. 1A: Effect of palbociclib on KYSE-30 and HEK cell viability after 48h exposure



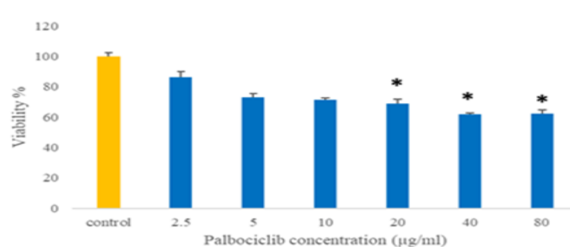


Figure No. 2B: Effect of palbociclib on KYSE-30 and HEK cell viability after 48h exposure

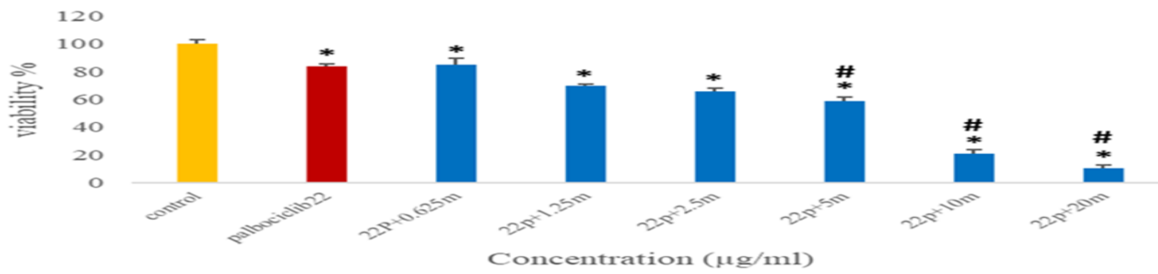


Figure No. 3C: Combination effect of fixed palbociclib (22 µg/mL) with varying melittin concentrations on KYSE-30 cell viability after 48h exposure

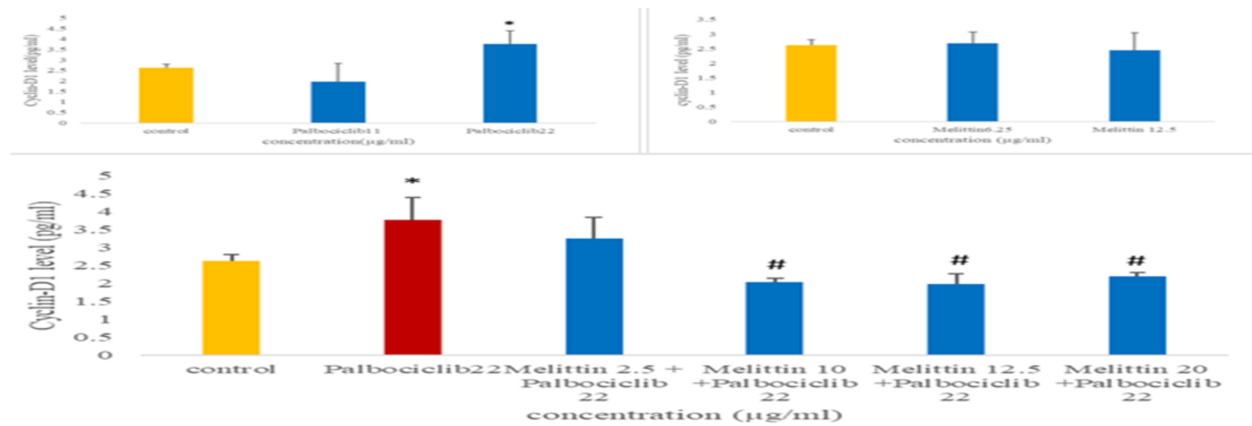


Figure No. 4A: Effect of palbociclib (11 and 22 µg/mL) on Cyclin-D1 level. Fig. 4B: Effect of melittin (11 and 22 µg/mL) on Cyclin-D1 level (Dunn's method). Fig. 4C: Effect of melittin and palbociclib combination on Cyclin-D1 level. Palbociclib (22 µg/mL) was used alone or in combination with melittin (2.5, 10, 12.5, and 20 µg/mL)

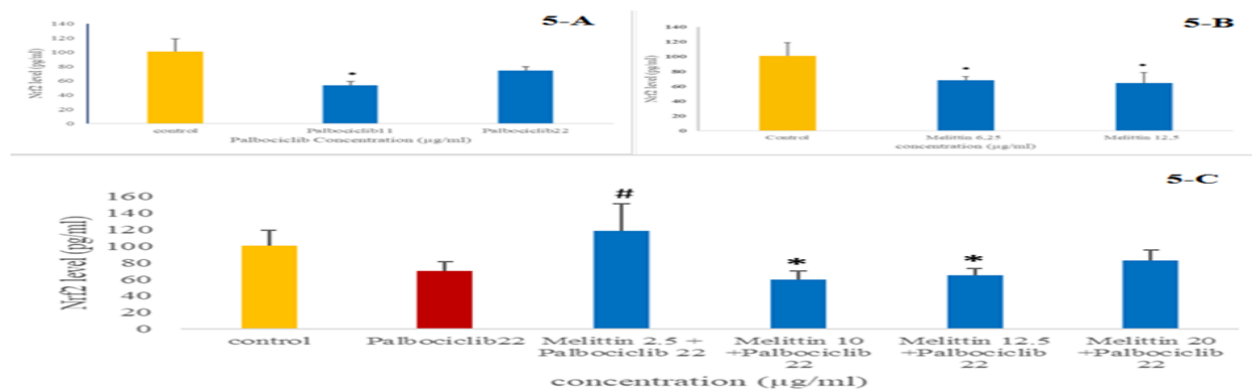


Figure No. 5A: Effect of palbociclib (11 and 22 µg/mL) on Nrf2 level. Fig. 5B: Effect of melittin (6.25 and 12.5 µg/mL) on Nrf2 level. Fig. 5C: Effect of melittin and palbociclib combination on Nrf2 level. Palbociclib (22 µg/mL) was used alone or in combination with melittin (2.5, 10, 12.5, and 20 µg/mL).

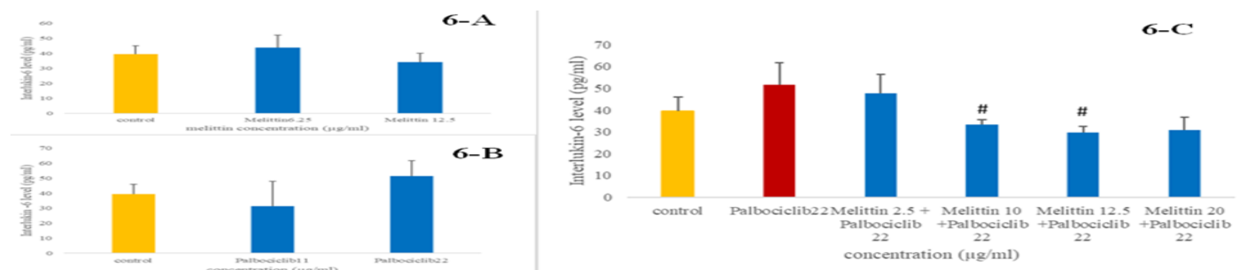


Figure No. 6A: Effect of melittin (6.25 and 12.5 µg/mL) on IL-6 level. Fig. 6B: Effect of palbociclib (11 and 22 µg/mL) on IL-6 level. Fig. 6C: Effect of melittin and palbociclib combination on IL-6 level. Palbociclib (22 µg/mL) was used alone or in combination with melittin (2.5, 10, 12.5, and 20 µg/mL)

Table No. 1: Wound closure over time measured in pixels

Groups	Wound with			% Closure at 48 h
	0 h	24 h	48 h	
Control	330	155.33	-	100%
Palbociclib 2.5 µg/mL	336.66	326	324	3.8%
Palbociclib 5 µg/mL	330	303.33	324	1.8%
Melittin 2.5 + Palbociclib 2.5	335.33	333.66	330.66	1.4%
Melittin 2.5 + Palbociclib 5	332.33	333.66	341.33	-2.7%
Melittin 5 + Palbociclib 5	333.33	340.66	344	-3.2%

DISCUSSION

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies with limited treatment choices and poor prognosis.[1,4] Although palbociclib, a selective CDK4/6 inhibitor, has shown efficacy in breast cancer, its monotherapy in ESCC has been largely ineffective due to intrinsic and acquired resistance mechanisms.[10,12] Melittin has the capacity to kill cancer cells through membrane damage and apoptosis.[14,15] Nevertheless, it can be used only be active at very high doses that might affect normal cells as well.[16]

The MTT assay demonstrated that melittin significantly reduced KYSE-30 cell viability at concentrations of 5 and 10 µg/mL after 24 hours, while showing toxicity to normal HEK cells only at the highest concentration tested (20 µg/mL). This selective cytotoxicity is consistent with previous reports indicating that melittin preferentially targets cancer cells due to their altered membrane composition and higher negative charge.[14,15] Zhu et al[15] previously demonstrated that melittin inhibited ESCC cell growth in a time- and dose-dependent manner with IC_{50} values of 1.88 µM for ECA109 and 1.64 µM for TE13 cells, and also showed that melittin enhanced radiation sensitivity in ESCC through induction of apoptosis.

Palbociclib significantly reduced KYSE-30 viability at concentrations of 20-80 µg/mL after 48 hours, with an IC_{50} of 22 µg/mL. However, palbociclib also reduced HEK normal cell viability at similar concentrations (20-80 µg/mL), indicating that palbociclib lacks selective toxicity between cancer and normal cells. the results obtained correspond to the mode of action of CDK4/6 inhibitors. CDK4/6 inhibitors block cell proliferation; however, they do not cause cell death directly. This effect is called cytostatic effect.[9,10]

The cells were treated with a fixed concentration of palbociclib (22 µg/mL) and various concentrations of melittin (0.625 to 20 µg/mL). The effect of all combinations on KYSE-30 cell viability was found to be significantly lower than the effect observed in the control group (p<0.001). Particularly, the combined treatment was effective in low concentrations of melittin that did not show any activity in monotherapy. In such a case, the combined effect of palbociclib and melittin is likely to be synergistic. It is possible that one mechanism of the synergy is the increased sensitivity of cancer cells after their cycle arrest by palbociclib, which enhances the ability of melittin to damage the membranes.

Cyclin-D1 serves as a regulatory protein and governs the G1-to-S phase transition during the cell cycle. ESCC cells typically show elevated expression of this protein. Our study revealed that palbociclib alone at 22 µg/mL caused an unexpected rise in Cyclin-D1 levels. This observation runs counter to the known action of CDK4/6 inhibitors, which usually reduce Cyclin-D1 via a feedback mechanism involving Rb and FOXM1.[9] This increase most likely reflects a compensatory adaptive response. Cancer cells boost Cyclin-D1 production to survive drug-induced pressure. Similar resistance patterns have been reported in other cancer models.[11]

In contrast, melittin by itself did not change Cyclin-D1 levels. This suggests that melittin's killing effect does not depend on cell cycle disruption. Earlier work supports this view, showing that melittin triggers ESCC cell death through Bcl-2 family protein modulation, not through cell cycle blockade.[15]

When combined, melittin (10, 12.5, and 20 µg/mL) with palbociclib (22 µg/mL) gave a different picture. The combination markedly lowered Cyclin-D1 expression. Hence, melittin corrected the palbociclib-driven increase, thereby countering a key resistance pathway.[9,13] It was absent at the smallest melittin concentration (2.5 µg/mL). This finding emphasizes that an ideal drug ratio is essential for synergy.[16]

Nrf2 is a transcription factor that regulates the antioxidant defense machinery of the cell. Many cancers including ESCC maintain high Nrf2 activity to avoid oxidative damage from chemotherapy. The important rise of Nrf2 levels with palbociclib at 11 µg/mL. Palbociclib effects are reduced by cancer cells activating antioxidant pathways. Such adaptive responses are known well to power drug resistance. Similar findings have been observed in previous studies of CDK4/6 inhibitors.[9,12] At the higher dose (22 µg/mL), palbociclib did not change Nrf2 and the injury was so severe that no effective defense could be made.

Melittin alone gave a reverse effect. It significantly decreased (P=0.024) Nrf2 level at 6.25 µg/mL and 12.5 µg/mL. This is in agreement with earlier studies showing that melittin disrupts membrane structure and

induces oxidative stress.[14,15] This behavior of melittin might avoid the usual resistance mechanisms.[17,18]

When given together, melittin (10 and 12.5 µg/mL) plus palbociclib (22 µg/mL) significantly lowered Nrf2 levels (P<0.05). Similar to Cyclin-D1, melittin reversed palbociclib induced Nrf2 upregulation. Not all combinations did this however. In contrast, melittin (2.5 µg/mL) plus palbociclib (22 µg/mL) increased the level of Nrf2. The result indicates the importance of the melittin dose. Since the findings are consistent across the use of two different molecular markers (Cyclin-D1 and Nrf2), they provide solid evidence that the synergistic action is indeed present. In light of Nrf2 induction being one of the factors leading to drug resistance, the ability of melittin to inhibit it can help overcome resistance to palbociclib.[13,16]

IL-6 is a pro-inflammatory cytokine. It promotes tumor development and metastasis and contributes to treatment resistance. These functions have been well-documented in ESCC and other malignancies. Melittin alone at 6.25 and 12.5 µg/mL did not cause any significant change in IL-6 levels. Also, palbociclib alone at 11 and 22 µg/mL had no effect on IL-6 levels. However, the combined therapy induced definitive effect, 10 µg/mL melittin with 22 µg/mL palbociclib has decrease the level of IL-6 (P=0.016), 12.5 µg/mL melittin with 22 µg/mL palbociclib has decrease the level of IL-6. High IL-6 levels are associated with worse outcomes and greater drug resistance in ESCC patients. Thus, lowering IL-6 is therapeutically useful. Beyond its direct cancer-killing activity, melittin may also act as an anti-inflammatory agent. Its ability to reduce IL-6 levels could enhance patient survival. Additionally, the melittin-palbociclib combination might decrease tumor-driven inflammation.

In the untreated control group, cells closed the gap entirely within 48 hours (100% closure). This reflects the typical migratory behavior of cancer cells. Palbociclib given alone strongly suppressed cell movement. At 2.5 µg/mL, the wound closure rate was just 3.8%. At 5 µg/mL, it was only 1.8%. These results show that palbociclib effectively blocks ESCC cell motility and cell migration represents a crucial step in the metastatic process.

The combination of melittin (2.5 µg/ml) and palbociclib (2.5 µg/ml) generated the same results, with a wound closure rate of 1.4%. This difference was not statistically significant compared to the use of palbociclib alone. The higher combined doses produced a different effect. The use of melittin (at a concentration of 2.5 µg/ml) with palbociclib (at a concentration of 5 µg/ml) resulted in a 2.7% decrease in wound healing. Using the same concentration also resulted in a 3.2% decrease. These negative ratios indicate that the wound is widening, not healing. That is, the size of the wound has increased over time. This is strong evidence of a cytotoxic effect. The combination not only prevented the movement of cells, but also killed them, causing the wound area to increase. The results strongly support the synergistic toxic effect of the combination of melittin and palbociclib.

CONCLUSION

The pharmacodynamic potentiation of melittin (10 µg/mL) in combination with palbociclib (22 µg/mL) on KYSE-30 esophageal cancer cells. The combination decreased cell viability, decreased Cyclin-D1 levels, suppressed the Nrf2 antioxidant defense, decreased IL-6 levels and blocked cell migration. These results suggest that melittin may block two major resistance mechanisms to palbociclib: cyclin-D1 feedback upregulation and Nrf2 activation. This combination could be a promising new treatment for esophageal squamous cell carcinoma (ESCC). However, these results need to be supported by further animal studies before clinical application.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Hayder Hasan Sabri, Reyadh H. Al-Mosawi
Drafting or Revising Critically:	Hayder Hasan Sabri, Kaiser N. Madlum
Final Approval of version:	All the above authors
Agreement to accountable for all aspects of work:	All the above authors

Conflict of Interest: The study has no conflict of interest to declare by any author.

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