

Melittin, A Potential Natural Toxin with Anticancer Properties: Regulating IL-1 β , COX-2 and TNF- α in Human Colorectal Cancer Cells WiDr

Makkasau Plasay^{1,*} and Lukman Muslimin²

¹Department of Nursing Pharmacology, STIKES Panakkukang, Makassar, South Sulawesi 90231, Indonesia

²Department of Pharmaceutical Chemistry, Sekolah Tinggi Ilmu Farmasi Makassar, South Sulawesi 90242, Indonesia

(*Corresponding author's e-mail: makkasau_mkes@yahoo.co.id)

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Abstract

Despite decades of study, the antiproliferation and molecular processes of the biomolecular elements of honeybee venom (i.e., melittin) as anticancer agents remain largely unclear. This study illustrated the antiproliferation effect of melittin by regulating IL-1 β , COX-2 and TNF- α in human colorectal cancer cells WiDr. In order to assess the antiproliferation, various concentrations of melittin were contacted to the WiDr cell through MTT assay. Moreover, the ability of melittin to regulate the expression of IL-1 β , COX-2 and TNF- α was determined by ELISA. The cytotoxic effect showed the inhibition of the WiDr cell in a dose-dependent manner, with the IC₁₀, IC₂₅ and IC₅₀ values of 0.095 ± 0.001 , 0.147 ± 0.002 and 0.207 ± 0.004 , respectively. Additionally, in further investigations, melittin markedly inhibited the IL-1 β and COX-2 expression but induced the TNF- α , demonstrating significant ($p < 0.05$) elevations compared to the control group. These findings point to the antiproliferative properties of melittin in WiDr cells. Therefore, as a unique natural treatment for colorectal cancer, melittin may have a lot of potential.

Keywords: Antiproliferation, Colorectal cancer, Cytokines, Melittin, Toxicity

Introduction

Globally, cancer is a serious public health issue. In 2019, an estimated 23.6 million (95 % UI, 22.2 - 24.9 million) new cases of cancer were reported, along with 10.0 million (95 % UI, 9.36 - 10.6 million) cancer deaths. There were also an estimated 250 million (235 - 264 million) disability-adjusted life years (DALYs) lost to cancer [1]. A common malignant neoplasm is colorectal cancer (CRC), which is determined by the information that is currently available. Its incidence ranges from being the second to the fourth most common type of cancer globally, depending on factors such as region, type of cancer and gender. Moreover, globally, CRC is third in terms of recognition (6.1 %) and second in terms of mortality (9.2 %). By 2035, rectal and colon cancer-related mortality are expected to rise by 60 and 71.5 %, respectively [2,3].

The immune system and its components are critical in the pathogenesis of CRC, as they are in the etiology of numerous cancers. Cytokines are multifunctional immune system mediators that play a role in both pro- and anti-inflammatory immune responses. Interleukin-1 (IL-1) is a member of an 11-member family that plays a role in inflammatory reactions [4]. IL-1 is generated and secreted by a variety of cell types inside the tumor, including immune cells, fibroblasts and cancer cells [5].

The control of COX-2 involves IL-1, which is crucial, as the concentrations of COX-2 and IL-1 are significantly correlated. Moreover, human mesangial cells' production of COX-2 is induced by IL-1 through a variety of signaling pathways, including PPAR β / δ /augments [6]. Furthermore, breast cancer has been shown to up-regulate the pro-inflammatory cytokine TNF, and high levels of TNF are linked to breast cancer recurrence [7]. In serous ovarian cancers, TNF levels also have a favorable correlation with tumor grade. Additionally, TNF-deficient mice are less prone to develop skin cancers when exposed to 7,12-Dimethylbenz(a)anthracene (DMBA) or 12-O-tetradecanoylphorbol-13-acetate (TPA) [8].

Many cancer patients in Indonesia opt for chemotherapy as the treatment for CRC. However, chemotherapy drugs frequently result in cancer cell resistance, which in most cases leads to therapeutic failure [9]. One of the potential future treatments for cancer is melittin, a significant peptide component of bee venom. According to recent studies, melittin can cause cytotoxicity in a variety of cancer cell types by

altering the cell cycle, inhibiting growth and/or proliferation, and inducing apoptosis as well as necrosis through several cancer cell death mechanisms, such as the activation of caspases and matrix metalloproteinases [10]. The cytotoxic effect of melittin inhibits MCF-7 cell growth in a dose-dependent manner, with an IC_{50} value of 5.86 g/mL (i.e., extremely toxic) for an antiproliferative effect against breast cell carcinoma MCF-7. When compared to control cells, the IC_{50} -treated cells clearly had higher levels of p53 and 8-OHdG [11].

Cell-based research is crucial for proving the effectiveness and mechanism of medications. For instance, in order to confirm the cytotoxic and apoptotic effects, Hamdan *et al.* [12] administered phycocyanin and *Citrullus colocynthis* extract to WiDr cells in proportion to the dose. Thus, our study aimed to verify the possibility of using melittin as a candidate for cancer therapy. First, we investigated the antiproliferation effect of melittin against WiDr cells. Afterward, the expression of the related components in the $IL-1\beta$, COX-2 and TNF- α signaling pathways and their levels were also analyzed.

Materials and methods

Study design

This study was a laboratory experimental study that used a post-test only control group design in between January and July 2022.

Materials

Melittin from honeybee venom was purchased from Sigma Aldrich (Catalog No. M2272), with purity of $\geq 85\%$ (HPLC) and molecular weight of 2,846.46 g/mol. Moreover, amphotericin B (250 μ g/mL), penicillin-streptomycin (5,000 units/mL), fetal bovine serum (FBS), trypsin-EDTA (0.25 %), DMEM and high glucose were purchased from Gibco, while sodium dodecyl sulfate, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and phosphate buffered saline pH 7.4 (PBS) were purchased from Sigma Aldrich. Additionally, by aseptic filtering with a sterile polyethylene sulfone filter membrane ($d = 0.22\ \mu$ m), the medium was sterilized and stored at 4 °C.

Cell culture

The human colorectal cancer cells WiDr were obtained from Hasanuddin University Medical Research Center (HUMRC). The cells were cultured in the DMEM medium supplemented by 10 % FBS, 1 % penicillin-streptomycin and 0.5 % amphotericin B. Moreover, the cells were maintained at 37 °C in a CO_2 incubator (5 % CO_2 ; 95 % humidified air). On the day before the experiment, the confluent cells were harvested by using 0.05 % trypsin-EDTA after they were washed with PBS.

Cell viability test

The ability of melittin to inhibit the proliferation of WiDr cells was determined by using MTT assay in the manner described by Tanumihardja *et al.* [13], although with a slight modification [13]. First, the cells were seeded onto a 96-well plate (Corning Life Science) at 10^5 cells/mL, before being incubated at 37 °C for 24 h. Then, the cells were washed with PBS, and 100 μ L of melittin was given to the cells at varied doses (0.02 - 0.32 μ M), while the control cell was given 100 μ L of medium. After 24 h of incubation, the medium was discarded and washed with PBS. Later, 100 μ L of MTT (0.5 mg/mL) was added to each well and then incubated for an additional 4 h. The live cell was metabolizing MTT to formazan, so 100 μ L of 10 % SDS was added to stop the reaction, and this was allowed 4 h in a dark room. Finally, using a microplate reader, the optical density (OD) of each well was measured at 595 nm. The following formula was used to manually compute the proportion of viable cells:

$$\text{Inhibition (\%)} = \frac{OD_{\text{control}} - OD_{\text{sample}}}{OD_{\text{control}}} \times 100$$

Moreover, IC_{10} , IC_{25} and IC_{50} were calculated in a straight line (i.e., linear regression).

Expression of $IL-1\beta$, COX-2 and TNF- α

The confluent cells were harvested, calculated (2×10^5 cells/mL) and transferred onto 24-well plate culture dishes (Corning Life Science). After 24 h of incubation, 500 μ L of melittin in the concentrations of IC_{10} , IC_{25} and IC_{50} were added, and the dishes were allowed to stand at 37 °C for 24 h. Additionally, for the purpose of measuring the synthesis of $IL-1\beta$, COX-2 and TNF- α expression, the culture supernatants were collected and centrifuged at 10,000 rpm for 5 min at 4 °C. Furthermore, the levels of $IL-1\beta$, COX-2

and TNF- α were measured in accordance with the guidelines given in the FineTest protocol kit with catalog numbers of EH0185, EH1014 and EH0302, respectively.

Statistical analysis

The data were presented as mean \pm SD for at least 3 independent experiments, and the difference between the groups was evaluated using ANOVA followed by Tukey's post hoc test, with a significance level of $p < 0.05$.

Results and discussion

Natural products are becoming more and more popular for their usage as medicinal supplements, either theoretically or experimentally, in addition to being used as dietary supplements. The main biological and pharmacological component of honeybee venom is called melittin. Its potent lipid membrane surface action [14], along with its anti-microbial [15], anti-inflammatory [16], muscle-injury-treating [17] and anticancer properties [10] have been widely studied and proven.

Melittin, which makes up around 50 % of all honeybee venom, is the main chemical in the substance. It has 26 amino acid residues with the exception of the C-terminal region, the majority of which have hydrophobic or at least neutral side chains [18]. At the moment, anticancer peptides are in the spotlight due to their easy synthesis, high biodegradability, decreased toxicity to healthy tissues, and, most crucially, decreased drug resistance. When compared to traditional medicines, anticancer peptides have emerged as a viable cancer therapy method [19,20].

Cytotoxicity of melittin on human colorectal cancer cells WiDr

Melittin's antitumor activity against WiDr was assessed using a culture tetrazolium test (MTT assay). Moreover, the inhibitory concentrations were determined from the dose-response curve by using different melittin concentrations. **Table 1** presents the outcomes of the assay employing melittin at various concentrations, ranging from 0.02 to 0.35 μM . These experimental results demonstrated that melittin, like doxorubicin, repressed WiDr cells in a dose-dependent manner. Additionally, melittin's IC_{50} value was $0.207 \pm 0.004 \mu\text{M}$, which was significantly lower than doxorubicin's IC_{50} value of $0.828 \pm 0.012 \mu\text{M}$, indicating that melittin is more powerful than doxorubicin.

Table 1 Toxicity effects of melittin and doxorubicin against WiDr cell after 24 h of incubation.

| Melittin | | Doxorubicin | |
|-------------------------------------|-------------------|---------------------------------|-------------------|
| Concentration (μM) | Inhibition (%) | Concentration (μM) | Inhibition (%) |
| 0.02 | 6.10 ± 1.39 | 0.09 | 1.00 ± 0.68 |
| 0.04 | 11.17 ± 3.03 | 0.18 | 3.06 ± 1.20 |
| 0.07 | 22.36 ± 2.91 | 0.37 | 9.64 ± 1.64 |
| 0.11 | 30.40 ± 1.89 | 0.55 | 14.20 ± 0.44 |
| 0.14 | 40.71 ± 0.89 | 0.74 | 24.43 ± 2.38 |
| 0.18 | 52.13 ± 0.07 | 0.92 | 36.23 ± 2.24 |
| 0.21 | 70.26 ± 3.34 | 1.10 | 47.08 ± 2.29 |
| 0.25 | 80.81 ± 2.28 | 1.29 | 72.53 ± 1.28 |
| 0.28 | 95.95 ± 0.04 | 1.47 | 81.57 ± 4.54 |
| 0.32 | 98.61 ± 2.40 | 1.66 | 95.83 ± 0.05 |
| 0.35 | 100.76 ± 0.49 | 1.84 | 99.59 ± 0.50 |
| Linearity (R^2) | 0.9949 | | 0.9923 |
| IC_{10} (μM) | 0.095 ± 0.001 | | 0.196 ± 0.011 |
| IC_{25} (μM) | 0.147 ± 0.002 | | 0.468 ± 0.023 |
| IC_{50} (μM) | 0.207 ± 0.004 | | 0.828 ± 0.012 |

Many studies have already investigated the anticancer properties of melittin. Its dose-dependent way against breast cancer MCF-7 cell with an IC_{50} value of $5.86 \mu\text{g/mL}$ was observed [11]. Moreover, Mansour *et al.* [21] reported the cytotoxicity effect of melittin on liver HepG2 cells. Furthermore, a dose-dependent anti-proliferative was found with an IC_{50} value of $78.38 \mu\text{g/mL}$. Similar to our findings, Askari *et al.* [15] in a recently published paper have reported that melittin exhibited cytotoxic activity with IC_{50} values $6.45 \mu\text{g/mL}$ human primary fibroblast cell line, C654. Furthermore, melittin was significantly more potent against human HER2-enriched breast cancer (SKBR3) with IC_{50} values $5.77 \text{ ng}/\mu\text{L}$ [10]. Considering this, the results of this study regarding antiproliferation were in line with the previous studies. Meanwhile, the IC_{50} value of melittin against the colon cancer WiDr cell was $0.207 \pm 0.004 \mu\text{M}$, indicating that the melittin possess is an anticancer agent.

Melittin reduces the production of IL-1 β by WiDr cells

The IL-1 β production in WiDr cells treated with melittin was quantified by ELISA. It was discovered that the levels of IL-1 dropped as melittin concentrations rose. In the case of IL-1 β , there was a decrease in its amount (**Figure 1**) even with the lowest concentration of melittin (IC_{10}), and the lowest decrease in the amount of IL-1 β production by IC_{50} of $17.82 \pm 0.29 \text{ pg/mL}$ was significant ($p < 0.05$) to all the groups ($p < 0.05$). It was found that IL-1 influences a wide range of physiological processes. It can control cellular adhesion and migration, angiogenesis, immunological response, gene expression and cytokine production [5]. Moreover, we demonstrated that IL-1 increased in a variety of solid tumors such as melanoma, colon, lung, breast, or head and neck cancers, and that this upregulation is linked to a worse prognosis. IL-1 was found in abundance in a mouse model of adenomatous polyposis coli colon cancer [22]. The apoptosis-associated speck-like protein was required to trigger the release of IL-1 after the melittin treatment [23].

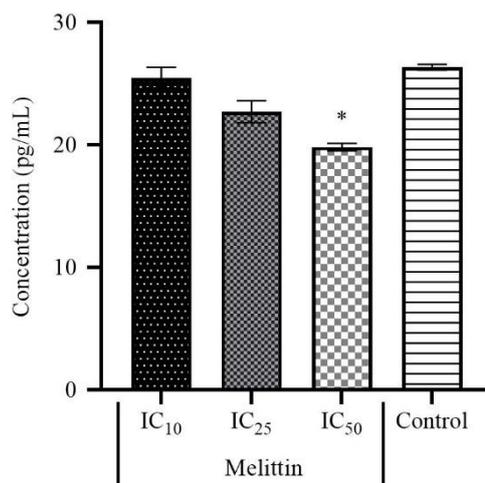


Figure 1 The effects of melittin on the expression of IL-1 β in WiDr cells; the cells were either left in culture media alone for 24 h or exposed to melittin at varied concentrations of IC_{10} , IC_{25} and IC_{50} . Moreover, the data were presented as mean \pm SD ($n = 3$). Furthermore, the difference between the groups was evaluated using ANOVA followed by Tukey's post hoc test, with a significance level of $p < 0.05$. Here, superscript (*) signifies to all groups.

Melittin reduces the production of COX-2 by WiDr cells

Furthermore, the most recent results also demonstrated that WiDr cell death is linked to the overexpression of IL-1. By suppressing the miR-101 expression through a COX-2/HIF1 pathway, IL-1 promotes cancer, while through its negative regulation of oncogene expression, MiR-101 prevents malignant transformation and the spread of cancer. As a result, the IL-1/miR-101 regulatory axis of pathogenic inflammatory signaling is new [24]. Early-stage mammary lesions are caused by a different mechanism, which is the IL-1-mediated production of COX-2 that has been identified [5].

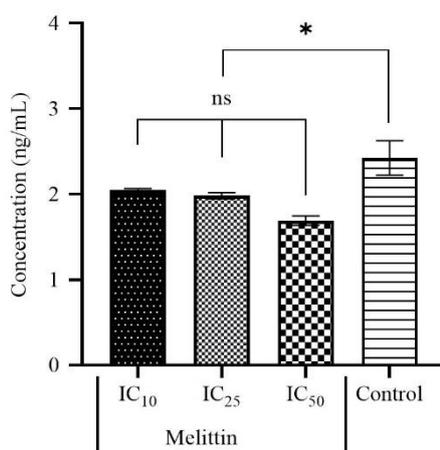


Figure 2 The effect of melittin on the expression of COX-2 in WiDr cells. The cells were either left in culture media alone for 24 h or exposed to melittin at varied concentrations of IC₁₀, IC₂₅ and IC₅₀. Moreover, the data were presented as mean ± SD (n = 3). Furthermore, the difference between the groups was evaluated using ANOVA followed by Tukey's post hoc test, with a significance level of $p < 0.05$. Here, superscript (*) signifies the control well, while (ns) no signifies.

Furthermore, in the case of COX-2, there was a decrease in its amount (**Figure 2**) even with the lowest concentration of melittin (IC₁₀ $0.095 \pm 0.001 \mu\text{M}$), whereas a high concentration of melittin (IC₅₀ $0.207 \pm 0.004 \mu\text{M}$) was required to bring down the amount of COX-2 produced. The production of COX-2 of WiDR cells after treatment with melittin was not significant ($p > 0.05$), but all the groups of melittin were significant to the control group ($p < 0.05$). Besides, the amount of COX-2 after contacting with IC₁₀, IC₂₅ and IC₅₀ were 2.07 ± 0.03 , 1.89 ± 0.17 and 1.78 ± 0.17 ng/mL, respectively, while the negative control was 2.42 ± 0.20 ng/mL.

Melittin enhances the production of TNF- α by WiDr cells

Afterward, by using ELISA, we examined the levels of TNF- α expression in WiDr cells, whether they were melittin-treated or not. As shown in **Figure 3**, the control cells expressed a lower concentration of TNF- α (29.17 ± 0.34 pg/mL), whereas the level of expression dramatically increased when the cells were stimulated with IC₅₀ of melittin. Therefore, it is clear that melittin increased IL-1 in a dose-dependent manner.

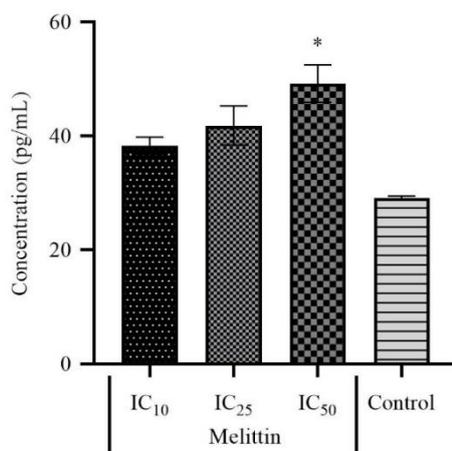


Figure 3 The effects of melittin on the expression of TNF- α in WiDr cells. The cells were either left in culture media alone for 24 h or exposed to melittin at varied concentrations of IC₁₀, IC₂₅, and IC₅₀.

Moreover, the data were presented as mean \pm SD ($n = 3$). Furthermore, the difference between the groups was evaluated using ANOVA followed by Tukey's post hoc test, with a significance level of $p < 0.05$. Here, superscript (*) signifies all groups.

Additionally, a significant ($p < 0.05$) increase in TNF expression was observed in the acquired data. A multifunctional cytokine called TNF plays a significant role in a variety of biological processes, including cell proliferation, differentiation and death. Since it is an inflammatory cytokine [25], TNF may mediate messages that are either pro-survival or pro-death. Particularly, in the context of T-cell-directed immunotherapies, the potential cytotoxicity of T-cell-produced TNF has generally been disregarded [26].

Conclusions

The study found a new therapeutic agent, melittin, a key peptide ingredient of bee venom, which can enhance human colorectal cell cancer. Hence, melittin showed cytotoxic activity against human colorectal cell cancer (WiDr) with an IC_{50} value was $0.207 \pm 0.004 \mu\text{M}$. We have provided evidence that the melittin may be induced the TNF- α production and inhibited IL-1 β and COX-2 expression that could play an important role in their cytotoxic activity. Further studies on the safety assessment must be evaluated to guarantee safety.

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