Anti-Cancer Activity of the Curcumin Analog Mono-O-Demethylcurcumin on Invasive Multidrug-Resistant Oral Squamous Cell Carcinoma in vitro

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Abstract

Metastasis is a major cause of death in cancer patients. Oral squamous cell carcinoma is the most common head and neck cancer, characterized by a poor prognosis and low survival rate. The average five-year survival rates vary from 50 to 80%, according to the occurrence of invasion and metastasis, which is responsible for determining the stage of the cancer. Curcuminoid is the major curcuminoid compound isolated from turmeric and has been reported to have excellent anti-cancer activity in various tumors in both in vitro and in vivo models. This study aimed to investigate the anti-cancer activity of the curcumin analog mono-O-demethylcurcumin compared with curcumin, in an invasive multidrug-resistant oral squamous cell carcinoma cell line (CLS-354/DX). The anti-proliferation effect was determined by tetrazolium dye MTT assay. The anti-migration and anti-invasion effects were investigated by the transwell migration and invasion chamber assay, respectively. The results showed that both compounds reduced CLS-354/DX viability and migration and invasion ability, but mono-O-demethylcurcumin was significantly more potent than curcumin. The half maximal inhibitory concentration (IC50) values of curcumin and mono-O-demethylcurcumin were 44.99±2.92 μM and 19.10±0.71 μM, respectively. Application of mono-O-demethylcurcumin and curcumin at non-cytotoxic concentrations inhibited cell migration and invasion in a dose-dependent manner. Treatment with 5 μM mono-O-demethylcurcumin decreased CLS-354/DX migration and invasion scores by 63.84±3.31% and 11.40±2.82%, respectively. While, treatment with 15 μM of curcumin showed increased CLS-354/DX migration and invasion scores by 72.45±4.17% and 21.70±4.39%, respectively. These results suggest that mono-O-demethylcurcumin exhibits excellent anti-proliferation, anti-migration and anti-invasion activity on multidrug-resistant oral squamous cell carcinoma. This compound may thus have a potential as a chemopreventative or therapeutic agent for multidrug-resistant oral cancer.

Keywords: anti-migration, anti-invasion, mono-O-demethylcurcumin, invasive multidrug-resistant oral squamous cell carcinoma

Introduction

Head and neck cancer, which is the sixth leading cancer worldwide, describes a group of cancers that originate in the lip, mouth, nose, throat, tonsil, pharynx, salivary gland and larynx [1, 2]. Oral cancer refers to cancers of the lip, tongue, gingiva, floor of the mouth, palate, maxilla, vestibule and retromolar area up to the anterior pillar of the fauces (1). Squamous cell carcinoma (SCC) is the most frequent malignant tumor of the head and neck region (2). Factors that are known to increase oral cancer risk include; use of tobacco, consumption of alcohol and betel quids containing areca nut, poor diet, physical inactivity, infection with high-risk types of human papillomavirus (HPV), and reproductive changes [2, 3]. Over 60% of patients with oral cancers present with either regional or distant spread [4]. Therefore, the five-year survival rates for oral cancer are poor, averaging between 50 to 80% and varying depending on the stage of the disease [5, 6]. The treatment approaches depend upon the stage and location of the cancer. Chemoradiotherapy is the established standard treatment for patients who present with inoperable cancers or patients in whom the operation would be associated with unacceptable morbidity. A pharmaceutical chemotherapy drug that is widely used to treat many solid tumors is cisplatin [7]. Cisplatin is a high responsiveness platinum-based chemotherapeutic agent, but its use is associated with multiple severe side effects affecting renal, otologic, and bone marrow function. Furthermore, the majority of oral cancer patients eventually relapse with cisplatin-resistant disease [8]. Therefore, the finding of a novel...
Chemotherapeutic agents and optimal chemotherapeutic treatment for oral cancer remains a challenge.

Curcuma longa, or turmeric, is a herbal plant that belongs to the family Zingiberaceae. Turmeric is comprised of a group of three major curcuminoids: curcumin (77%), demethoxycurcumin, (17%) and bisdemethoxycurcumin (3%) [9]. Compounds from C. longa have been reported to have several anti-cancer properties and curcumin has been reported to inhibit cell proliferation and promote apoptosis of various cancers, both in vitro and in vivo [10, 11]. Moreover, several anti-migration and anti-metastasis activities of curcumin have been reported, for example, curcumin inhibits invasiveness and epithelial-mesenchymal transition in oral squamous cell carcinoma by reducing matrix metalloproteinase 2, 9 and modulating the p53-E-cadherin pathway [12], curcumin inhibits the invasion of lung cancer cells by modulating the PKCα/Nox-2/ROS/ATF-2/MMP-9 signaling pathway [13], curcumin inhibits lung cancer invasion and metastasis by attenuating the GLUT1/MT1-MMP/MMP2 pathway [14], curcumin inhibits tumor epithelial-mesenchymal transition by downregulating the Wnt signaling pathway and upregulating NK2D expression in colon cancer cells [15], curcumin inhibits the invasion of thyroid cancer cells via down-regulation of the PI3K/Akt signaling pathway [16]. In addition, since the structure of β-diketone of curcumin can be easily catalytically decomposed, several curcumin analogs have been developed that show more potent anti-oxidant, anti-bacterial, anti-mycobacterial, anti-cancer, and anti-metastatic activity [17-21].

The aims of this study were to evaluate the anti-migration and anti-invasion activity of mono-O-demethylcurcumin (curcumin analog) in an invasive multidrug-resistant oral squamous cell carcinoma cell line (CLS-354/DX) compared with curcumin. Therefore, the impact on %viability, %migration and %invasion were evaluated.

Methodology

Chemicals and reagents
Mono-O-demethylcurcumin (curcumin analog) and curcumin compounds from curcuma longa were kindly supported by Prof. Dr. Apichart Suksamrarn, Faculty of Science, Ramkhamhaeng, University, Thailand and Dr.

CLS-354/DX invasive multidrug-resistant oral squamous cell carcinoma cell line was established from CLS-354 cell line (Cell Line Service, Germany) by Dr. Tanyarat Utaipan, Prince of Songkla University, Pattani Campus, Thailand and Asst. Prof. Dr. Warangkana Chunglok, Walailak University, Thailand. CLS-354/DX is fibroblast-like human mouth carcinoma cell line (or EMT-derived phenotype), which is more aggressive, as described previously [22]. This cell was maintained in a monolayer culture in RPMI-1640 medium with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine at 37°C with 5% CO2/ 95% air. CLS-354/DX cell line was grown as monolayer and cells were considered ready to treat when they reached 80% confluence.
Cells Viability Assay
CLS-354/DX cell line was seeded onto 96-well plates (Corning®, Sigma-Aldrich Pte Ltd., Singapore) with 1.5x10^6 cells per well in RPMI-1640 media with 10% FBS and cultured for 24h. After that cells were treated with various doses of mono-O-demethylcurcumin and curcumin. After 24h of treatment, cell viability was assessed by MTT assay. First, 0.5 mg/ml of MTT reagent was added to each well for at least 3 hours of treatment. Optical density (OD) values were determined at 560 nm and 670 nm using a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific Inc., CA, USA). The viable cell number was directly proportional to the production of formazan, which was reflected by the color intensity measured after solubilization with DMSO. All samples were assayed in triplicate in at least 3 independent experiments and results were calculated as half maximal inhibitory concentration (IC_{50}) as mean ± SEM by Graphpad prism 5. Student t-test analysis was used for statistical analysis.

Migration Chamber Assay
CLS-354/DX cells were resuspended to 1x10^4 cells/well in serum reduced medium containing mono-O-demethylcurcumin and curcumin at non-cytotoxic concentrations and were carefully transferred into the upper chambers of a Boyden migration chamber (Cell Biolabs, Inc., CA, USA). The lower chamber was filled with 10% FBS medium. The Boyden migration chamber was incubated at 37°C with 5% CO₂ for 24 h. After that non-invading cells were removed with a cotton swab moistened with medium and invested cells were fixed by methanol for 2 min. Invested cells were stained with crystal violet stain solution for 2 min and counted in 10 randomly selected microscopic fields (20x). Experiments were performed three times and results expressed as the invasive ability ± SEM. Values were calculated by Graphpad prism 5 and Student t-test analysis was used for statistical analysis. The migration chambers were warmed to room temperature and incubated at 37°C with based culture medium for 2 hours after remove the package from -20°C.

Results
Mono-O-demethylcurcumin inhibited CLS-354/DX cell proliferation
CLS-354/DX cells were oral squamous cell carcinoma cells line exhibiting elongated (fibroblast-like) shape. The cells are invasive and multidrug resistant [23]. The methylthiazoletetrazolium (MTT) assay was performed to measure the inhibitory effect of mono-O-demethylcurcumin on tumor cell proliferation (Figure 2A). The methylthiazoletetrazolium (MTT) assay was performed to measure the inhibitory effect of mono-O-demethylcurcumin on tumor cell proliferation (Figure 2A). CLS-354/DX cell line was seeded onto 96-well plates and treated with various concentrations of mono-O-demethylcurcumin and curcumin. A range of 0–60 µM of compounds was used to treat CLS-354/DX cell line for 24 h. After that the half maximal inhibitory concentration (IC_{50}) was calculated. Mono-O-demethylcurcumin demonstrated satisfactory inhibitory activities against the CLS-354/DX cell line. The half maximal inhibitory concentration value (IC_{50}) of mono-O-demethylcurcumin was 19.10±0.71 µM, while the IC_{50} value of curcumin against this cell line was 44.99±2.92 µM (Table 1). Morphological observation showed that both 19 µM of mono-O-demethylcurcumin and 40 µM of curcumin could reduce cell viability by approximately 50% following the 24h treatment (Figure 2B). These results therefore demonstrated that mono-O-demethylcurcumin exhibits better anti-proliferation activity of CLS-354/DX than curcumin and cisplatin (Table 1). In addition, the non-cytotoxic and 10% inhibitory concentration (IC_{10}) were calculated for subsequent experiments. Results showed that IC_{10} concentration of mono-O-demethylcurcumin was approximately 5 µM and IC_{10} concentration of curcumin was 15 µM. At this concentration, the anti-proliferative effect was not evident (Figure 2A).
Figure 2 (A) Proliferation effects of mono-O-demethylcurcumin, curcumin, cisplatin (positive control) and vehicle control (dimethyl sulfoxide) on CLS-354/DX cell line. CLS-354/DX cell line was incubated with mono-O-demethylcurcumin (3.7, 7.5, 15, 30 and 60µM) for 24h and performed by the MTT assay. Optical density values were measured at 560nm and 670nm wavelength. All samples were performed in triplicates at least 3 independent experiments and results were calculated %cell viability as mean ± SEM by Graphpad prism 5. Student t-test analysis was used for statistical analysis.

*p< 0.05 compared with control group (Untreated cell), ***p< 0.001 compared with control group (Untreated cell).

(B) Cell morphology of CLS-354/DX cell upon treated with IC50 of mono-O-demethylcurcumin (19µM), curcumin (40µM), vehicle control (1.2µM) and untreated control for 24 h.

Table 1 IC50 and IC10 of mono-O-demethylcurcumin on CLS-354/DX cell line.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (µM)</th>
<th>IC10 (µM)</th>
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<tbody>
<tr>
<td>Cisplatin</td>
<td>30.21±2.76</td>
<td>10</td>
</tr>
<tr>
<td>Curcumin</td>
<td>44.99±2.92a</td>
<td>15</td>
</tr>
<tr>
<td>Mono-O-demethylcurcumin</td>
<td>19.10±0.71ab</td>
<td>5</td>
</tr>
</tbody>
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IC50: half maximal inhibitory concentration.
IC10: 10% inhibitory concentration.
Data were expressed as mean ± SEM (n= 5-11). Student t-test analysis was used for statistical analysis. a: differences are statistically significant when compare with cisplatin, b: differences are statistically significant when compare with curcumin

**Mono-O-demethylcurcumin inhibited CLS-354/DX cell migration**

The effect of mono-O-demethylcurcumin on cell migration was investigated in the CLS-354/DX cell line by using a transwell migration chamber assay. The CLS-354/DX cell line was seeded onto the upper chamber with IC\textsubscript{10} quantities of mono-O-demethylcurcumin and curcumin in serum reduced medium and the lower chamber contained 10% FBS medium. After incubation at 37°C, 5% CO\textsubscript{2} for 24h, the number of migrated cells were counted and cell migration rate was calculated as percent of control. Moreover, the dose-dependent effects of compounds were also evaluated. The results showed that both mono-O-demethylcurcumin and curcumin inhibited CLS-354/DX cell migration in a dose-dependent manner. Treatment with 5µM and 10µM of mono-O-demethylcurcumin decreased CLS-354/DX migration by 63.85±3.3% and 31.20±2.91%, respectively. On the other hand, treatment with 15µM and 30µM of curcumin decreased CLS-354/DX migration by 72.45±4.17% and 33.55±2.12%, respectively (Figure 4). The comparison between mono-O-demethylcurcumin and curcumin revealed that mono-O-demethylcurcumin exhibited better anti-migration activity in CLS-354/DX than curcumin (Figure 5).

**Figure 4** Concentration-dependent inhibitory effects of mono-0-demethylcurcumin-treated and curcumin-treated on CLS-354/DX cell migration. The graphs represent CLS-354/DX cell migration ability after 24 h treatment with 5µM and 10µM mono-0-demethylcurcumin and 15µM and 30µM curcumin. Values were calculated by Graphpad prism 5. Data are reported as mean ± SEM, n= 3. Student t-test analysis was used for statistical analysis. **p< 0.001 compared with control group (Untreated cell), #p value< 0.001 compared with lowest concentration of group. The photographic image (20x) represents the number of migrated CLS-354/DX cells.
Figure 5 Comparison migration inhibitory effects of mono-O-demethylcurcumin and curcumin in CLS-354/DX cell line. The graphs represent CLS-354/DX cell migration ability after 24 h treatment with IC\textsubscript{10} concentrations of mono-O-demethylcurcumin (5µM) and curcumin (15µM). Values were calculated by Graphpad prism 5. Data are reported as mean ± SEM, n= 3. Student t-test analysis was used for statistical analysis. ***p< 0.001 compared with control group (Untreated cell). The photographic image (20x) represents the number of migrated CLS-354/DX cells.

Mono-O-demethylcurcumin inhibited CLS-354/DX cell invasion

The effects of mono-O-demethylcurcumin on CLS-354/DX cell invasion ability were investigated by using IC\textsubscript{10} concentrations with a standard invasion chamber assay. Concentrations of 5µM of Mono-O-demethylcurcumin and 15µM of curcumin were added into the upper compartment of the invasion chamber. After incubation at 37°C, 5% CO\textsubscript{2} for 22h, the number of invasive cells was counted and the cell invasion rate was calculated as percent of control. The results showed that mono-O-demethylcurcumin decreased number of CLS-354/DX invasion about 11.40±2.82% While, curcumin decreased number of CLS-354/DX invasion about 21.70±4.39% (Figure 6).
Figure 6 Invasion inhibitory effects of mono-O-demethylcurcumin and curcumin in CLS-354/DX cell line. The graphs represent CLS-354/DX cell invasion ability after 22 h treatment with IC$_{10}$ concentrations of mono-O-demethylcurcumin (5µM) and curcumin (15µM). Results expressed as the mean invasive ability ± SEM microscopic field. Values were calculated by Graphpad prism 5. Student t-test analysis was used for statistical analysis. ***p< 0.001 compared with control group (Untreated cell). The photographic image (20x) represents the number of invasive cells was determined using an invasion chamber assay.

Discussion and Conclusions

*Curcuma longa*, (*C. longa*) or turmeric, is comprised of a group of three major curcuminoids; curcumin (77%), demethoxycurcumin, (17%) and bisdemethoxycurcumin (3%). Curcumin is the main constituent found in the rhizomes of the plant. Polyphenols are responsible for the medicinal effects of these compounds [9, 24]. Mono-O-demethylcurcumin is a very minor natural curcuminoid. However, Mono-O-demethylcurcumin can be synthesized by removal of a methyl group from curcumin by demethylation with a 43% yield of synthesis [25]. Numerous previous studies have provided evidence of the anti-carcinogenic properties of curcumin in many cancers, both *in vitro* and *in vivo*, including oral and head and neck cancers [9]. Curcumin inhibits cell proliferation and induces cell cycle arrest and apoptosis in various cancer cells i.e. prostate, breast, pancreatic, lymphoma and oral squamous cell carcinoma, by targeting multiple pathways [26-29]. The half maximal inhibitory concentration values (IC$_{50}$) of curcumin in invasive cancer cell lines are between 20-40 µM [19, 30, 31]. In this study, the IC$_{50}$ of curcumin in an invasive multidrug-resistant oral squamous cell carcinoma cell line (CLS-354/DX) was about 45 µM. This high inhibitory dose of curcumin limits its use in therapeutic application so curcumin analogs have attracted considerable attention. Curcumin analogs have shown potential anti-cancer effects and have induced cell apoptosis better than parental curcumin [15-19]. Compared with curcumin, curcumin analogs have better metabolic stability and pharmacological activity [17]. In this study, mono-O-demethylcurcumin (curcumin anolog) exhibited better anti-proliferation activity, as evaluated by MTT assay, than curcumin. Multidrug resistance (MDR), including cisplatin resistance, is the most critical problem leading to therapeutic failure of head and neck cancer. The IC$_{50}$ of cisplatin in the multidrug resistant CLS-
354/DX cell line in our study was 33.23±0.96µM, which is consistent with previous reports [23]. In the current study, the results also show that mono-O-demethylcurcumin exhibited a better anti-proliferative effect than the standard drug cisplatin. Moreover, mono-O-demethylcurcumin appears to have limited cytotoxicity to normal cells. Mono-O-demethylcurcumin was nontoxic to a human gingival fibroblast cell line at concentrations up to 10µg/mL or 28.22µM [32]. In an immortalized rat microglial cell line, mono-O-demethylcurcumin at concentrations of 20–40µM could decrease cell viability significantly in a concentration dependent manner. However, at concentrations of 10µM viability was similar to the untreated control [33].

Multiple organ failure caused by metastasis is a major cause of death in cancer patients. In fact, prognosis of cancer is mainly determined by the invasiveness of the tumor and its ability to metastasize. Although there are several drugs available to control cancer growth in humans, there are no drugs presently available to specifically inhibit the metastasis of cancer cells. Thus, active compounds demonstrating anti-invasive and anti-metastatic properties are defined as a new catalog of chemopreventive agents [34, 35]. Curcumin has been reported to inhibit invasiveness, metastasis and epithelial-mesenchymal transition in many cancer cell lines such as oral, lung, thyroid and colon cancer cell line through a number of different cellular pathways. For example, curcumin inhibits lung cancer invasion and metastasis by attenuating the GLUT1/MT1-MMP/MMP2 pathway [14], curcumin inhibits tumor epithelial-mesenchymal transition by downregulating the Wnt signaling pathway and upregulating NKD2 expression in colon cancer cells [15], curcumin inhibits the invasion of thyroid cancer cells via down-regulation of the PI3K/Akt signaling pathway [16]. However, there were are few reports about anti-invasiveness and anti-metastasis activity of curcumin analogs, and there are no reports about the anti-invasiveness and anti-metastasis activity of mono-O-demethylcurcumin in invasive oral squamous cell carcinoma. In this study, mono-O-demethylcurcumin (curcumin analog) was found to inhibit the migration and invasion activity of invasive multidrug-resistant oral squamous cell carcinoma evaluated by migration and invasion chamber assays. However, the underlying mechanism still needs further study to be elucidated.

Taken together, the results in this study suggest that mono-O-demethylcurcumin exhibited excellent anti-cancer activity on invasive multidrug-resistant oral squamous cell carcinoma. Mono-O-demethylcurcumin could possess anti-proliferation, anti-migration and anti-invasion activity against other cell lines. Although, further study is required to evaluate both the underlying mechanism and to evaluate the anti-cancer activity in an in vivo study, these results show that mono-O-demethylcurcumin has potential as a chemotherapeutic agent for multidrug-resistant oral cancer.

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References


