Biological Evaluation of Curcumin As a Natural RANKL Inhibitor in Osteosarcoma

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Abstract: Osteosarcoma is an important health problem among children and teenagers. Metastases is observed in about twenty percent of osteosarcoma patients. RANK/RANKL/OPG signaling pathway is involved in formation of osteosarcoma and its metastases. Especially, the overexpression of RANKL is related to osteoclast and osteosarcoma formation. Therefore, inhibition of RANKL activity has been significant treatment method of osteosarcoma. In this study, curcumin was evaluated as RANKL inhibitor in osteosarcoma cells. In this context, anti-proliferative and anti-invasive properties of the curcumin were determined with in-vitro and computational assays in Saos-2 cell line. Obtained results showed that curcumin may have a potential for treatment of osteosarcoma and its metastases.

1. INTRODUCTION

Osteosarcoma is the most common type of cancer that originates in the bone and it is diagnosed cancer types of 3% of children. Osteosarcoma is the most common malignancy in bone and 3% of diagnosed cancer types are osteosarcoma in children. Age, gender, radiation, bone infarct and genetic susceptibility may increase more likely for developing osteosarcoma. Osteosarcoma usually develops in osteoblasts and it can metastasize to parts of the skeleton and other tissues especially lung in later stage of disease. The 5-year survival rate of people with osteosarcoma is about 70%, although this rate is average 20% of patients with metastasis. Chemotherapy is common treatment method in osteosarcoma before and after surgery. Unfortunately, current therapy methods are no effective and target specific drugs are not available to treat osteosarcoma yet [1-3]. Therefore, the understanding of molecular mechanisms of the osteosarcoma is essential for designing target specific therapeutic agents.

RANK/RANKL/OPG signaling pathway plays significant roles in bone biology and osteosarcoma (Figure-1). RANKL (receptor activator of nuclear factor-xB ligand) is produced by osteoblasts and binds to RANK (receptor activator of nuclear factor-xB) selectively. RANKL is responsible for osteoclastogenesis and stimulates osteoclast formation and bone resorption. Furthermore, RANK is involved in differentiation and activation of osteoclasts. Apart from RANK and RANKL, OPG (osteoprotegerin) is produced by osteoblast cells and inhibits RANK-RANKL interaction. Thus, OPG prevents osteoclast formation and bone resorption. The balance of RANKL/OPG ratio is important parameter in bone homeostasis. RANKL is overexpressed in osteosarcoma cells and the increase of
RANKL/OPG ratio is the most important biological marker in osteosarcoma. Therefore, designing target specific compounds for inhibition of RANKL activity is significant approach to treatment of osteosarcoma [4-7].

**Figure 1.** Structure of RANK-RANKL (A) and OPG-RANKL (B) (RANK: Magenta, OPG: Green, RANKL: Orange)

Curcumin is well-known herbal supplement which is produced from Curcuma longa plants (Figure-2). Curcumin exhibit good tolerability and bioavailability properties; therefore, curcumin has been characterized as “Generally Recognized as Safe” (GRAS) by US Food and Drug Administration (FDA). Generally, supplementation of curcumin inhibits inflammation markers and induces presences of endogenous antioxidants in the body. Up to now, a wide variety of biological activities of the curcumin including anticancer, antioxidant, and antiviral, have been determined in pre-clinical and clinical studies. Numerous studies indicated that curcumin inhibits cell proliferation and blocks a variety of cellular signaling pathways in cancer cells [8-11].

**Figure 2.** Structure of curcumin

In this study, anticancer activity of curcumin was determined on human osteosarcoma cell line (Saos-2) along with molecular docking and proteomic experiments. Our results report curcumin may be potent drug candidate as RANKL inhibitor in treatment of osteosarcoma.

2. MATERIALS AND METHODS

2.1. Materials

Saos-2 cell line was from ATCC (American Type Culture Collection, USA). Modified McCoy’s 5A medium, heat-inactivated fetal bovine serum, L-glutamine, trypsin–EDTA, gentamycin and XTT ((2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cell proliferation kit were supplied from Biological Industries Ltd. Human RANK, RANKL and OPG ELISA kits were from YL Biont Ltd. Curcumin was purchased from Sigma Aldrich.

2.2. Cell Culture

To perform in-vitro experiments, human osteosarcoma Saos-2 cells were cultured in modified McCoy’s 5A medium supplemented with 10% fetal bovine serum and 0.1% gentamycin. Cells were cultivated in 5% CO2 at a temperature of 37°C.

2.3. Cell Proliferation Assay

Saos-2 cells were seeded in 96-well plates at 1x10^4 cells per well and incubated overnight. On the following days, the osteosarcoma cells were exposed to curcumin between 200-0.012 µM concentrations with two-fold dilution for 48 hours. To measure cell viability, XTT cell proliferation kit was applied according to instructions from the supplier. The IC50 value of curcumin on Saos-2 cells was calculated with GraphPad Prism 6.0 software [12,13].

2.4. Cell Migration Assay

Wound healing assay was performed to investigate anti-invasive activity of curcumin on Saos-2 cells. Osteosarcoma cells were seeded in 6-well plate and cells were incubated until they covered approximately 90% of the plate surface. Then, cell monolayers were scratched by yellow pipette tip and cells were washed with PBS. Curcumin was incubated with scratched cells for 48h and cell images were photographed at 0h, 24h and 48h with Olympus CKX53 inverted microscope to measure the closure ratio of the wound area. The migration levels of the cells in effect of the curcumin were calculated according to control.

2.4. Molecular Docking

To determine molecular mechanism of interaction between curcumin and RANK-RANKL, docking calculations were performed with Docking Server (http://www.dockingserver.com/web) [13,14]. The 3D structural coordinates of RANK-RANKL complex (PDB code: 3ME2) was retrieved from Protein Data Bank (www.rcsb.org), and the 2D structure of curcumin was obtained from PubChem. Before docking calculation, heteroatoms of the protein complex were removed and grid box of the complex was determined as 45, 47, and 60 Å for x, y and z respectively. PyMol visualization software was used to analyze docking results.

2.5. RANK, RANKL and OPG Protein Level Assay

The differences RANK, RANKL and OPG expression levels with the effect of curcumin on Saos-2 cells were measured by commercial ELISA kits according to the manufacturers’ instructions. Curcumin was incubated with Saos-2 cells at 48h and cells were washed with PBS twice. Then, cells were lysed using cold-ice buffer
solution (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.2 mM PMSF, pH:7.4) and obtained cell lysates were stored at -80 °C to perform ELISA assays. The total protein concentrations of the cell lysates were determined by Bradford method [15].

2.6. Statistical Analysis

Differences in the mean values of measured activities were evaluated statistically using the SPSS 17.0 program with one-way analysis of variance (ANOVA). The results were represented as a mean ± standard error of the mean (SEM). Probability values of p < 0.05 were considered to be significant.

3. RESULTS AND DISCUSSION

3.1. Cell Proliferation Assay

To determine the anti-proliferative activity of curcumin on osteosarcoma cells, Saos-2 cells were treated with curcumin by using a variety of concentrations for 48h. Before XTT experiment, curcumin was dissolved in DMSO and diluted with medium to contain 0.1% DMSO. According to the XTT assay results, IC_{50} value of curcumin was calculated as 12.7 µM on Saos-2 cells and osteosarcoma cells exposed to curcumin displayed a significant reduction in cell proliferation in a dose-dependent manner compared with control. Curcumin contains both methoxy (-OCH3) and hydroxyl (-OH) groups in aromatic rings. It was important to note that methoxy groups generate an excess of reactive oxygen species (ROS) and stimulate apoptotic and autophagic pathways in cancer cells. Also, methoxy groups may enhance lipophilicity of the compound, and this makes it easier to uptake the compound into the cell [14]. The presence of hydroxyl group increase water solubility of compounds and influence the electron density of the phenyl ring [16]. These factors may positively contribute to the interaction of curcumin and RANK-RANKL complex.

3.2. Cell Migration Assay

To investigate anti-invasive properties of the curcumin in osteosarcoma, Saos-2 cells were incubated with curcumin for 48h. The wound area of control and curcumin treated Saos-2 cells were calculated as approximately 60% and 90% (p<0.05) respectively after 24h. At the end of the 48h, the wound areas were determined about 25% and 75% (p<0.05) in control and curcumin treated osteosarcoma cells respectively (Figure-3). Obtained results demonstrated that curcumin exhibited anti-invasive activity at 24h and 48h against osteosarcoma cells. Metastases are diagnosed in one out of every five patients with osteosarcoma and survival rate of patients was decreased dramatically in metastatic cases. Therefore, the development of dual-effect drugs is significant pharmaceutical approach to inhibit proliferation and invasion processes of osteosarcoma. In this study, curcumin blocked both cell proliferation and invasion process of osteosarcoma. Thus, curcumin may be potent drug template to inhibit cell proliferation and metastasis in osteosarcoma.

3.3. Molecular Docking

Molecular docking calculations were performed for better understanding of the interaction between curcumin and RANK-RANKL protein complex. Table-1 shows the docking analysis results and Figure-4 presents the interaction between RANK-RANKL complex and curcumin.

![Figure 3](image3.png)

**Figure 3.** Migration assay results of curcumin and control in Saos-2 cells (C: Control, S: Curcumin)

![Figure 4](image4.png)

**Figure 4.** A) Interaction of curcumin (shown in green) with RANK-RANKL complex (RANK: magenta, RANKL: orange). B) Specific interactions of curcumin (shown in magenta) with RANK-RANKL (Residues and curcumin are shown in green and magenta sticks model, respectively)

<table>
<thead>
<tr>
<th>Est. Free Energy of Binding (kcal/mol)</th>
<th>Est. Inhibition Constant (K*)</th>
<th>vDW+Hbond desolv Energy (kcal/mol)</th>
<th>Electrostatic Energy (kcal/mol)</th>
<th>Total Interaction Energy (kcal/mol)</th>
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<tbody>
<tr>
<td>-6.94</td>
<td>44.02 µM</td>
<td>-7.42</td>
<td>-0.44</td>
<td>-7.47</td>
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According to the docking results, curcumin selectively bond to interface region of RANK-RANKL complex (Figure-4) with -6.94 kcal/mol estimated free energy of binding and 44.02 µM estimated inhibition constant (Table-1). Curcumin was localized to the ligand binding cavity of the RANK-RANKL complex and interacted with Lys97, Asn147, Trp121, Cyc113, Leu99, Lys91, His252 and Arg112. These residues are found in ligand binding cavity of the RANK-RANKL complex. Especially, curcumin generated hydrogen bond with Lys97 (3.3 Å) and pi-pi interaction with Trp121. These
interactions may cause conformational changes in RANK-RANKL protein complex and thus, tumorigenesis of osteosarcoma is inhibited with the result of decreased RANKL activity in Saos-2 cells.

3.4. RANK, RANKL and OPG Protein Level Assay

RANK/RANKL/OPG is a key signaling pathway in osteosarcoma. Particularly, increasing RANKL and decreasing OPG protein expression levels are the most important clinical markers in patients with osteosarcoma. Aberrant expression of RANKL stimulates osteoclast formation, although up-regulation of OPG inhibits RANK-RANKL interaction and osteolysis. Therefore, down-regulation of RANKL/OPG is a significant success criterion for target specific drugs in treatment osteosarcoma [5,7].

Table 2. Protein levels of RANK, RANKL and OPG in curcumin-treated and non-treated Saos-2 cells (Data are presented as mean ± SEM, *p<0.05 compared to control)

<table>
<thead>
<tr>
<th></th>
<th>RANK (ng/ml)</th>
<th>RANKL (ng/ml)</th>
<th>OPG (ng/ml)</th>
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<tr>
<td>Control</td>
<td>0.87 ± 0.05</td>
<td>4.72 ± 0.14</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td>Curcumin</td>
<td>0.85 ± 0.03</td>
<td>1.97 ± 0.09*</td>
<td>0.84 ± 0.09*</td>
</tr>
</tbody>
</table>

In this study, down-regulation of RANKL and up-regulation of OPG are determined with curcumin-treated osteosarcoma cells in compared to control group (Table-2, p<0.05). Furthermore, RANKL/OPG ratio decreased from 9.25 to 2.34 in osteosarcoma cells with curcumin. Protein expression level of the RANK has not changed significantly in curcumin-treated Saos-2 cells compared to control (p>0.05). Actually, the differences of RANK expression level are not proven biological marker in tumorigenesis of osteosarcoma (Table-2).

4. CONCLUSION

Osteosarcoma is important type of the bone cancer and survival rate of patients with osteosarcoma is low since there are no target specific drugs. RANK/RANKL/OPG may be target signaling pathway to develop efficient therapeutics against osteosarcoma. RANKL is overexpressed in osteosarcoma cells and down-regulation of RANKL blocks osteoclast formation and tumorigenesis of the osteosarcoma. Curcumin is a small-molecular weight natural compound which exhibits anti-tumoral activity in wide variety of cancer types. Curcumin interacts with RANK-RANKL, interference and decreases RANKL/OPG, ratio, and thus, it inhibits proliferation and invasion of osteosarcoma cells. Based on the results from this study, curcumin may promise drug candidate to treat osteosarcoma and its metastases. Also, the development of curcumin analogs may have great potential in treatment of osteosarcoma in future.

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