RESEARCH PAPER

# Cephalotaxine Modulates Osteogenic Differentiation And Bone Resorption Signalling Through Bmp-2/Runx2/Opg-Rankl Axis In Human Osteosarcoma Mg-63 Cells

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**Abstract**

**Background:** Osteosarcoma is a primary bone malignancy, which is aggressive, and it is associated with the dysregulation of osteogenic signalling and bone remodeling. Aiming at the molecular regulators of osteogenesis and tumor proliferation is a good treatment approach. We have analysed the antiosteosarcoma properties of the natural alkaloid, cephalotaxine in the given study using an integrated methodology that involved cellular, molecular, and computational studies.

**Objective:** This research examined the molecular mechanisms that facilitate the therapeutic effects of cephalotaxine, a natural alkaloid, on human osteosarcoma mg-63 cells.

**Methods:** Mineralization potential was evaluated by alizarin red s staining and alkaline phosphatase (alp) histochemistry. The expression of targeted gene was quantified with real-time pcr. Western blotting was used to study protein expression of igf-ir and osteogenic markers.

**Results:** Cephalotaxine significantly inhibited the formation of mineralised nodules and calcium deposition. Gene and protein analyses demonstrated the downregulation of runx2, alp, osteocalcin, rankl, and igf-ir, in addition to modulation of opg expression.

**Conclusion:** Cephalotaxine reveals the anti-osteosarcoma potential by suppressing proliferative signalling and aberrant osteogenic differentiation through multiple pathways of molecular regulation. These findings demonstrate that cephalotaxine as a potential medication for the therapeutic management of osteosarcoma.

**Keywords:** Bmp-2 Signalling, Cephalotaxine, Osteosarcoma, Osteogenic Differentiation, Runx2.

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## INTRODUCTION

Osteosarcoma, also known as Osteogenic sarcoma, is the predominant primary malignant bone tumour originating from primitive osteoid-producing mesenchymal cells (1,2). It mostly occurs in children and young adults, primarily affecting the distal femur, proximal tibia, and

humerus, and is characterised by its aggressive and metastatic nature. Approximately one-fourth of patients with osteosarcoma develop metastatic lesions in the lungs (3). It is identified by aggressive growth, metastasis, and abnormal osteoid formation. This condition emerges sporadically; yet, over 70% of tumour tissues have chromosomal abnormalities. Additionally, the control of the cell cycle has been shown to exhibit hereditary abnormalities in some cases (4,5).

An increase in cell division, which is necessary for bone elongation, is the hallmark of osteosarcoma. During this process, cells may go through a number of alterations, such as deletion of tumour suppressor genes, which eventually causes them to become cancerous. Several conditions have also been linked to an increased risk of osteosarcoma in paediatric patients. These include retinoblastoma, Li-Fraumeni syndrome, and Rothmund-Thomson syndrome. Sarcomatous transformation is an uncommon consequence that happens to elderly persons with Paget's disease of the bone, could result in Osteosarcoma, in which the cancer no longer mostly affects the long bones, Instead, the jaw and pelvis are the areas that are most severely affected (6-8). Currently, the primary therapies for osteosarcoma are Surgical resection and conventional adjuvant chemotherapy. Osteosarcoma, on the contrary aspect, spreads and comes back quickly, which makes it more challenging for people to live. Tragically, one out of every five patients are diagnosed with metastasised osteosarcoma right away (3). In the molecular level, Osteosarcoma pathogenesis encompasses the dysregulation of several essential pathways of signalling which regulate bone homeostasis. RUNX2, the main regulator of osteoblastogenesis, is abnormally over-expressed in Osteosarcoma and facilitates malignant cells to invade and metastasise.

Bone morphogenetic protein-2 (BMP-2) and Alkaline Phosphatase (ALP) facilitate osteogenic differentiation, while the RANK/RANKL/OPG axis is essential for the regulation of osteoclast activation and tumor-induced osteolysis. In addition, the insulin-like growth factor 1 receptor (IGF-IR) and transforming growth factor-beta 1 (TGF-β1) serve as essential mediators of osteosarcoma cell proliferation, survival, and chemoresistance. Tumour progression and pathological mineralisation is facilitated by the dysregulation of osteogenic transcription factors like RUNX2, BMP-2, and TGF-β1. The RANK/RANKL/OPG axis is also very important in the bone remodelling imbalance that occurs in osteosarcoma. So, we need novel therapies for osteosarcoma that work promptly.

Natural alkaloids have become an important source of biologically active compounds exhibiting multifaceted pharmacological properties associated to cancer therapy. Cephalotaxine (CET) is a plant-derived alkaloid since it has a variety of different bioactivities and a unique chemical structure. Cephalotaxine and its esterified derivatives have been promising in the fight against microbial diseases, viral infections, as well as various types of malignancies since they were first discovered in the 1960s. Cephalotaxus hainanensis, or occasionally Cephalotaxus drupacea, is a Chinese coniferous tree that is the original source Cephalotaxine (CET) (9). Cephalotaxus harringtonia, a coniferous plant in the Taxaceae family which produces cephalotaxine and various other compounds as secondary metabolites. Cephalotaxine is one of these plant metabolites which is known for having an ideal composition that have been tried in clinical investigations. The leukaemia cell line shown significant susceptibility to the potent inhibitory effects of cephalotaxine-type alkaloids on several human cancer cell lines. The Food and Drug Administration (FDA) issued approval for homoharringtonine, a cephalotaxine ester, to be administered for the treatment of chronic myeloid leukaemia in 2012 (10-13). Cephalotaxine has shown anti-proliferative effects in several malignancies; however, its mechanistic influence on osteosarcoma and tumor-associated mineralisation is not yet fully elucidated.

The present study integrates with gene and protein expression profiling to elucidate the mechanistic foundation of cephalotaxine's effect by suppressing the Aberrant Osteogenic Signalling and IGF-1R Activation in osteosarcoma cells (MG-63) through direct molecular targeting. This work elucidated the mechanisms behind cephalotaxine’s potential as a low-toxicity phytotherapeutic agent for osteosarcoma by merging biochemical and cellular methodologies.

## MATERIALS AND METHODS

### MG-63 Cell Culture and Compound

Human osteosarcoma (MG-63) cells were obtained from the National Centre for Cell Sciences (NCCS) in Pune, India. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% foetal bovine serum (FBS) until reaching confluence. Penicillin (100 U/ml) and streptomycin (100 µg/ml) were incorporated into the medium to inhibit bacterial contamination. The culture medium containing cell lines was preserved in a humidified atmosphere containing 5% $CO_2$ at 37°C. The cells were then separated using trypsin-EDTA and used for

subculture or therapy. Cephalotaxine was purchased to prepare a stock solution and stored, then it is utilized for the following experiments.

**Cell Viability Assay (MTT)**

The MTT colorimetric cell viability assay was used to see how Cephalotaxine influenced MG-63 human osteosarcoma cells. This test assesses mitochondrial dehydrogenase activity as a sign of metabolically active cells that are viable. To conduct the test, MG-63 cells were put into 96-well flat-bottom culture plates at a density of 1 × $10^4$ cells per well in 100 µL of complete DMEM. They were then incubated overnight at 37°C in a humidified environment with 5% $CO_2$ to enable the cells to fully adhere and spread before treatment. After being attached overnight, the cells were treated with Cephalotaxine at different concentrations: control, 2.5, 5, 10, and 20 µM. The 10 mM DMSO stock solution was diluted in complete DMEM right before use, making sure that the final DMSO concentration did not go over 0.1% (v/v) in any treatment group. The cells were treated for 48 hours at 37°C in 5% $CO_2$. The vehicle control wells had the same amount of DMSO-containing media as the other wells, but without Cephalotaxine. After 48 hours of treatment, the culture media was carefully aspirated, and each well was gently washed twice with 1× phosphate-buffered saline (PBS) to get rid of any leftover drug and dead cell debris. The MTT solution was then put into each well and kept at 37°C in 5% $CO_2$ for 4 hours. During this time, metabolically active live cells used the mitochondrial succinate dehydrogenase enzyme to turn the yellow MTT tetrazolium salt into insoluble purple formazan crystals. After incubation, the medium with MTT was carefully removed by aspiration. To dissolve the formazan crystals completely, 100 µL of pure DMSO was added to each well and gently shaken on a plate shaker for 10 minutes at room temperature. Using a microplate reader, the absorbance of the purple formazan solution was measured at 495 nm.

**Mineralization assay**

Matrix mineralisation has been evaluated by Alizarin Red S staining method. The Alizarin red S dye adheres to calcium salts in cells, and the mineralisation of MG-63 cells has been quantified utilising spectrophotometric analysis. ALP staining was carried out with napthol ASMX as the substrate. For the quantitative analysis, alizarin red-stained cells were treated with 100 mM cetylpyridinium chloride for 1 hour to solubilise and liberate calcium-bound alizarin red into the solution, followed by spectrophotometric measurement of absorbance at 570 nm. The absorbance readings correlate strongly with the degree of matrix mineralisation. Alizarin Red S staining demonstrates how much calcium was actually deposited to the matrix, the physical end-product of mineralization by staining $Ca^{2+}$ ions red and dissolving the dye in cetylpyridinium chloride so that it can be measured at 570 nm. ALP Staining evaluates how active the osteoblastic differentiation machinery by examining the red azo dye complex that forms when ALP breaks down naphthol ASMX in the presence of fast red BB salt.

**Gene Expression analysis by Real Time-PCR**

The present investigation's gene expression analysis consisted of quantifying mRNA transcripts of important osteogenic and bone resorption-related genes, such as RUNX2, Osteocalcin, Osteoprotegerin (OPG), Alkaline Phosphatase (ALP), and Receptor Activator of Nuclear Factor κB Ligand (RANKL), as well as confirming their respective protein expression in Cephalotaxine-treated human osteosarcoma MG-63 cells. The procedure got started with the extraction of total RNA using TRIzol reagent, according to the phenol-chloroform extraction principle. This was followed by isopropanol precipitation and a wash with 70% ethanol to obtain pure RNA, which was then dissolved in nuclease-free water and measured spectrophotometrically. The reverse transcription of 1.5 µg of total RNA to complementary DNA (cDNA) was carried out using the cDNA Synthesis Kit (Qiagen, Hilden, Germany), therefore transforming the extracted mRNA transcripts into stable cDNA for downstream amplification. Real-Time Quantitative PCR (qRT-PCR) carried out through quantity-One-Software (Bio-Rad, Hercules, CA, USA) was used to measure the transcript levels of all target genes. Beta-actin (β-actin) or GAPDH was used as the housekeeping reference gene to adjust for variations in RNA input and cDNA synthesis efficiency among samples. List of primers employed in this study are mentioned in the Table 1.

**Table 1: Primers employed in this study**

| Gene | Forward primer | Reverse Primer | ize (bp) | Accession No. |
|---|---|---|---|---|
| ALP | ACCTCGTTGACACCTGGAAG | CCACCATCTCGGAGAGTGAC | 189 | NM_000478 |
| Runx2 | ACCTCGTTGACACCTGGAAG | TCTCGGTGGCTGGTAGTGA | 309 | NM_010150 51 |
| IGF1R | TGGGGAATGGAGTGCTGTAT | ACGTTTGGCCTCCCTGAACG | 438 | NM_012918 58.2 |

| | | | | |
|---|---|---|---|---|
| **OCN** | GAATGGTCACTGGGCTGTTT | CTGGCAGCTTTGCACAATTA | 109 | NM_199173.4 |
| **GAPDH** | GGAGTCAACGGATTTGGT | ATCAATGGAAATCCCATCAC | 206 | NM_002046 |

Primer design was carried out for all six gene targets, and the annealing temperature was optimised to provide consistent amplification conditions. The specificity of the reaction was confirmed using melt curve analysis, which also confirmed the efficiency of the reaction and the performance of the primers. Western blot analysis of RUNX2, ALP, BMP-2, and Osteocalcin proteins confirmed the RT-PCR results at the protein level. This added to the transcript analysis by showing how the transcriptional upregulation seen at the mRNA level was linked to the translational changes observed at the protein level in Cephalotaxine-treated MG-63 cells.

**Protein Expression by Western Blot**

Western blotting was employed to examine at the effect of Cephalotaxine on the expression of important osteogenic and bone resorption-related proteins in control and Cephalotaxine-treated MG-63 human osteosarcoma cells. These proteins included RUNX2, Alkaline Phosphatase (ALP), Bone Morphogenetic Protein-2 (BMP-2), Osteocalcin, and Insulin-like Growth Factor-1 Receptor (IGF-IR). Following treatment, cells were washed twice with ice-cold 1× phosphate-buffered saline (PBS) and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer with protease inhibitor and phosphatase inhibitor to stop proteins from breaking down during extraction. Cell lysates were incubated with occasional vortexing and then centrifuged to pellet cell debris. The resultant supernatant, which included all of the cellular protein, was carefully collected into new microcentrifuge tubes. Total protein concentration in each lysate was determined. Equal quantities of protein were denatured by boiling in sample buffer containing β-mercaptoethanol. The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on polyacrylamide gels for stacking and resolving. Finally, they were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, USA). After being transferred, membranes have been stopped with 5% non-fat dried skimmed milk powder suspended in Tris-buffered saline to prevent non-specific antibody binding. Following that, the membranes were left at 4°C overnight with the primary antibodies diluted. After being left overnight, the membranes were washed and then probed with suitable horseradish peroxidase (HRP)-conjugated secondary antibodies. The Enhanced Chemiluminescence (ECL) detection kit (Sigma, St. Louis, MO, USA) was used to observe protein bands and capture chemiluminescent signals. To make sure that all the lanes had the same amount of protein, beta-actin (β-actin) was utilised as the internal loading control. Using densitometry, the band intensities were measured, then normalised them to the β-actin band intensity, and reported them as relative protein expression.

**Statistical Analysis**

The present research's experimental data was obtained from three independent experiments that were each performed three times (n = 3). The data is shown as the mean ± standard deviation (SD). GraphPad Prism software version 8.0 was used to do the statistical analysis. One-way ANOVA followed by Tukey's post-hoc test was implemented to determine statistically significant differences between the control and Cephalotaxine-treated groups for gene expression levels from Real Time-PCR, protein expression levels from Western blot densitometric analysis, and matrix mineralization data from Alizarin Red S quantification. In this study, a probability value of $p < 0.05$ was always regarded statistically significant.

## RESULTS

**Cephalotaxine Reduces MG-63 Cell Viability in a Dose-Dependent Manner**

The inhibitory and cytotoxic effects of Cephalotaxine on MG-63 human osteosarcoma cells were quantitatively evaluated using the MTT cell viability test after treatment with increasing doses of 0, 2.5, 5, 10, and 20 µM. Figure 1A shows that Cephalotaxine caused a substantial and gradual dose-dependent reduction in the viability of MG-63 cells at all treatment doses compared to the untreated control. The untreated control cells (Group I) maintained elevated baseline vitality. At a concentration of 2.5 µM, Cephalotaxine treatment caused a modest but statistically significant fall in cell viability. This suggests that it has early antiproliferative effects at this sub-cytotoxic level. At 5 µM, there was a more noticeable and substantial drop in cell viability, with viable cells dropping below the untreated control ($p < 0.05$). At 10 µM and 20 µM, there were even bigger drops in cell viability, which showed that higher doses of Cephalotaxine continued to lower the survival rate of MG-63 cells in a dose-dependent way. Using GraphPad Prism 8.0, the estimated $IC_{50}$ value of Cephalotaxine against MG-63 cells was around 8–10 µM. This was based on the sigmoidal dose-response curve and non-linear regression analysis.

Figure 1A. Effects of Cephalotaxine on cell viability and morphology of MG-63 human osteosarcoma cells. Graph

showing cell viability percentage of MG-63 cells treated with Cephalotaxine at 0 (Group I/Control), 2.5, 5, 10, and 20 µM as determined by MTT assay. Absorbance was measured at 570 nm using a microplate reader. Data are expressed as mean ± SD from three independent experiments performed in triplicate (n = 3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test.

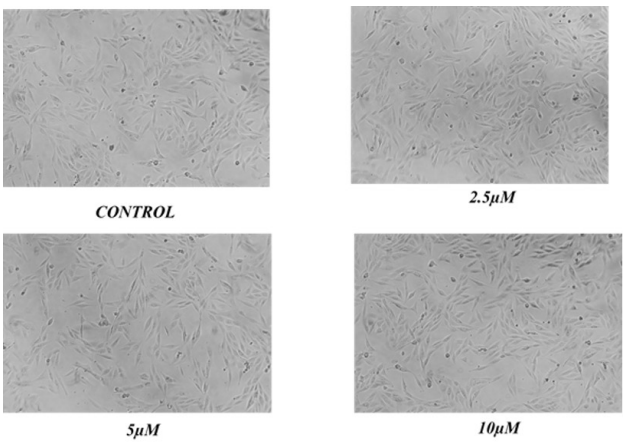


Figure 1B. Morphological analysis of MG-63 cells confirmed the MTT viability results and demonstrated concentration-dependent modifications in cell morphology and density across all treatment groups. These quantitative MTT and qualitative morphological results together show that Cephalotaxine significantly and concentration-dependently lowers the viability of MG-63 osteosarcoma cells.

**Effects of Cephalotaxine on RUNX2 mRNA expression in Human Osteosarcoma Cells (MG-63 cells)**

The effect of Cephalotaxine on the mRNA expression of RUNX2 which is the master transcription factor governing osteoblastic differentiation and a critical molecular driver of osteosarcoma progression and metastasis was evaluated by q-RT-PCR in MG-63 human osteosarcoma cells. As illustrated in Figure 3, RUNX2 mRNA expression was significantly upregulated in Cephalotaxine-treated MG-63 cells in a clear, progressive, and statistically significant dose-dependent manner compared to the untreated control group. The corresponding amplification curves demonstrated well-defined sigmoidal amplification profiles reaching a maximum plateau, with the Cephalotaxine-treated groups displaying visibly earlier threshold cycle (Ct) values relative to the untreated control curves, directly corroborating the greater RUNX2 mRNA abundance in treated cells. These findings collectively demonstrate that Cephalotaxine potently and dose-dependently activates RUNX2 transcription in MG-63 osteosarcoma cells, suggesting significant induction of osteoblastic differentiation signalling at the transcriptional level.

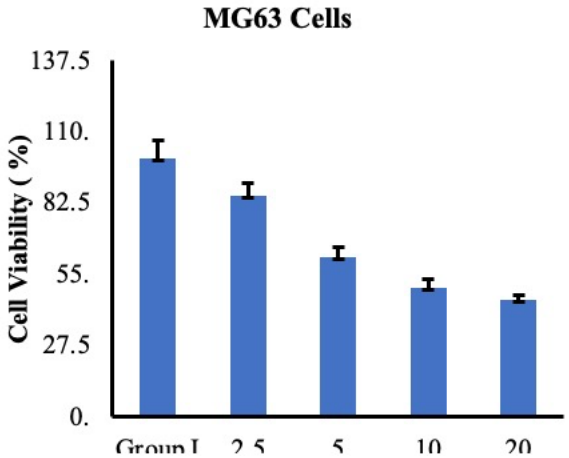


**Figure 3.** Effects of Cephalotaxine on RUNX2 mRNA expression of osteogenic differentiation and bone resorption-associated genes in MG-63 human osteosarcoma cells analysed by real-time quantitative PCR. In untreated control and Cephalotaxine-treated MG-63 cells (CEPHT 5 µM and CEPHT 10 µM), normalised to β-actin internal reference gene and expressed as fold change relative to untreated control. Right panels show corresponding representative amplification curves with fluorescence intensity (RFU) plotted against PCR cycle number. Data are expressed as mean ± SD from three independent experiments performed in triplicate (n = 3). Statistical significance between groups was determined by one-way ANOVA followed by Tukey's post-hoc multiple comparison test; $p < 0.05$ was considered statistically significant.

**Effects of Cephalotaxine on OPG mRNA Expression in Human Osteosarcoma Cells (MG-63 Cells)**

The effect of Cephalotaxine on the mRNA expression of OPG (Osteoprotegerin) which is a soluble decoy receptor belonging to the TNF receptor superfamily that competitively binds and neutralizes RANKL, thereby serving as a critical endogenous inhibitor of osteoclast differentiation, activation, and bone resorption was quantified by qRT-PCR in MG-63 human osteosarcoma cells following Cephalotaxine treatment. As demonstrated in Figure 4, OPG mRNA expression was significantly upregulated in a dose-dependent manner in Cephalotaxine-treated MG-63 cells relative to the untreated control group. At the higher concentration of Cephalotaxine, OPG mRNA expression was significantly elevated, which was also statistically significant confirming a progressive and concentration-dependent increase in OPG transcriptional upregulation. The amplification curves (right panel) demonstrated characteristic sigmoidal amplification profiles with plateau fluorescence reaching, and the staggered distribution of curves across cycles 20–40 reflected the varying Ct values between control and

treatment groups, validating the differential OPG mRNA expression levels. The significant dose-dependent upregulation of OPG mRNA by Cephalotaxine indicates potent transcriptional activation of this central anti-osteoclastogenic regulator, suggesting that Cephalotaxine may protect against pathological bone resorption in the osteosarcoma microenvironment through enhanced OPG-mediated neutralisation of RANKL.

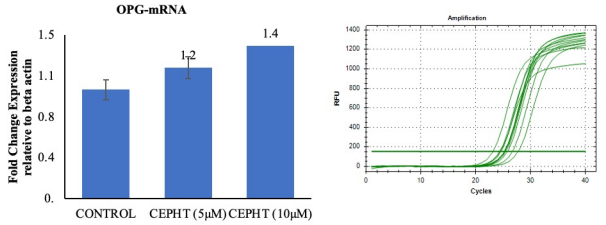


**Figure 4.** Effects of Cephalotaxine on OPG mRNA expression in MG-63 human osteosarcoma cells analysed by real-time quantitative PCR. In untreated control and Cephalotaxine-treated MG-63 cells (CEPHT 5 µM and CEPHT 10 µM), normalized to β-actin internal reference gene and expressed as fold change relative to untreated control. Right panels show corresponding representative amplification curves with fluorescence intensity (RFU) plotted against PCR cycle number. Data are expressed as mean ± SD from three independent experiments performed in triplicate (n = 3). Statistical significance between groups was determined by one-way ANOVA followed by Tukey's post-hoc multiple comparison test; $p < 0.05$ was considered statistically significant.

**Effects of Cephalotaxine on ALP mRNA Expression in Human Osteosarcoma Cells (MG-63 Cells)**

The transcriptional effect of Cephalotaxine on Alkaline Phosphatase (ALP) which is a membrane-bound glycoprotein enzyme that functions as an established early-to-mid stage marker of osteoblastic differentiation and plays a functional role in extracellular matrix mineralization by hydrolysing inorganic pyrophosphate to generate free phosphate ions essential for hydroxyapatite crystal nucleation and growth was evaluated by qRT-PCR in MG-63 human osteosarcoma cells. As shown in Figure 5, ALP mRNA expression was significantly and dose-dependently upregulated in Cephalotaxine-treated MG-63 cells compared to the untreated control group. At the higher concentration of Cephalotaxine, ALP mRNA expression was significantly and substantially elevated, which was also statistically significantly confirming a clear and stepwise dose-dependent increase in ALP transcriptional upregulation with escalating Cephalotaxine concentration. The amplification curves for ALP (right panel) demonstrated well-defined sigmoidal amplification profiles reaching a plateau with the amplification curves appearing at relatively later cycle numbers compared to RUNX2, consistent with the comparatively lower baseline abundance of ALP mRNA in MG-63 cells. The significant dose-dependent upregulation of ALP mRNA expression by Cephalotaxine at both 5 µM and 10 µM, corroborated by the corresponding ALP protein upregulation demonstrated by Western blot analysis and the enhanced ALP enzymatic activity confirmed by ALP histochemical staining, collectively establishes robust three-level transcriptional, translational, and functional evidence for Cephalotaxine-mediated induction of osteoblastic differentiation in MG-63 osteosarcoma cells.

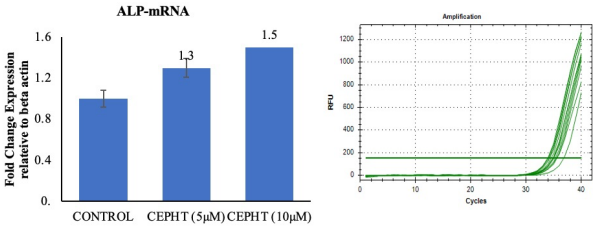


**Figure 5.** Effects of Cephalotaxine on ALP mRNA expression of osteogenic differentiation and bone resorption-associated genes in MG-63 human osteosarcoma cells analysed by real-time quantitative PCR. Right panels show corresponding representative amplification curves with fluorescence intensity (RFU) plotted against PCR cycle number.

**Effects of Cephalotaxine on RANKL mRNA Expression in Human Osteosarcoma Cells (MG-63 Cells)**

The effect of Cephalotaxine on the mRNA expression of RANKL (Receptor Activator of Nuclear Factor κB Ligand) which is a transmembrane member of the TNF superfamily expressed by osteoblasts and osteosarcoma cells that serves as the principal inducer of osteoclast differentiation, function, and survival, thereby acting as a critical driver of pathological bone resorption and tumor-induced osteolysis in osteosarcoma. As illustrated in Figure 6, RANKL mRNA expression was significantly downregulated in Cephalotaxine-treated MG-63 cells in a clear, progressive, and dose-dependent manner relative to the untreated control group. Treatment with Cephalotaxine at the higher concentration, RANKL mRNA expression was further and significantly reduced of the untreated control level ($p < 0.05$), confirming progressive and concentration-dependent transcriptional inhibition of RANKL with increasing Cephalotaxine concentration. The amplification curves for RANKL (right panel) demonstrated sigmoidal amplification profiles reaching a plateau at later cycle numbers, with the Cephalotaxine-treated groups displaying visibly delayed Ct values relative to the untreated control curves, directly reflecting

the reduced RANKL mRNA abundance in treated cells. Cephalotaxine may potently suppress pathological osteoclast-mediated bone destruction and tumor-induced osteolysis in the osteosarcoma microenvironment through dual and opposing transcriptional modulation of both OPG and RANKL.

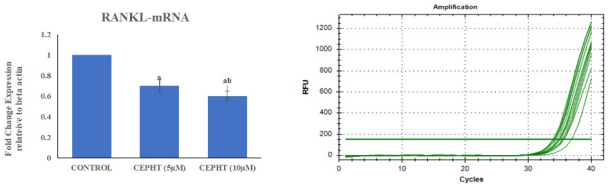


**Figure 6.** Effects of Cephalotaxine on RANKL expression of osteogenic differentiation and bone resorption-associated genes in MG-63 human osteosarcoma cells analysed by real-time quantitative
PCR. Right panels show corresponding representative amplification curves with fluorescence intensity (RFU) plotted against PCR cycle number.

## Effects of Cephalotaxine on Osteocalcin mRNA Expression in Human Osteosarcoma Cells (MG-63 Cells)

The effect of Cephalotaxine on the mRNA expression of Osteocalcin (OCN) which is the most abundant non-collagenous bone matrix protein exclusively synthesised and secreted by fully mature osteoblasts, serving as the most specific and definitive late-stage terminal marker of osteoblastic differentiation, bone matrix formation, and mineralization completion was evaluated by qRT-PCR in MG-63 human osteosarcoma cells. As demonstrated in Figure 7, Osteocalcin mRNA expression was significantly and substantially upregulated in Cephalotaxine-treated MG-63 cells in a dose-dependent manner relative to the untreated control group. At the higher concentration of Cephalotaxine, Osteocalcin mRNA expression was significantly elevated to the untreated control ($p < 0.05$), confirming a clear progressive dose-dependent increase in Osteocalcin transcriptional activation with escalating Cephalotaxine concentration. The amplification curves for Osteocalcin (right panel) demonstrated well-separated and clearly distinguishable sigmoidal amplification profiles reaching a plateau, with the Cephalotaxine-treated groups, particularly the 10 µM group displaying clearly earlier Ct values and faster amplification onset relative to the untreated control curves. The significant dose-dependent upregulation of Osteocalcin mRNA which is the most specific terminal marker of mature and fully differentiated osteoblasts in Cephalotaxine-treated MG-63 osteosarcoma cells.

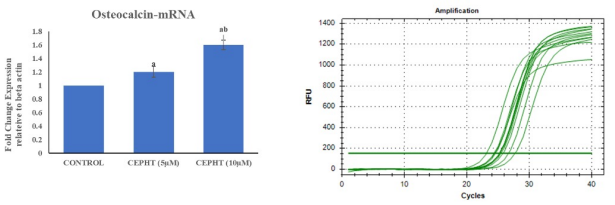


**Figure 7.** Effects of Cephalotaxine on Osteocalcin expression of osteogenic differentiation and bone resorption-associated genes in MG-63 human osteosarcoma cells analysed by real-time quantitative PCR. Right panels show corresponding representative amplification curves with fluorescence intensity (RFU) plotted against PCR cycle number.

## Effects of Cephalotaxine on Protein Expression Analysis

Western blot analysis was performed to evaluate the effect of Cephalotaxine on the protein expression of four key osteogenic differentiation markers such as RUNX2, Alkaline Phosphatase (ALP), Bone Morphogenetic Protein-2 (BMP-2), and Osteocalcin in MG-63 human osteosarcoma cells following treatment with Cephalotaxine at 5 µM and 10 µM. Protein band intensities were quantified by densitometric analysis, normalized to the corresponding β-actin loading control band intensity in each lane to correct for any variation in protein loading, and expressed as relative expression values compared to the untreated control group. Beta-actin was a reliable and stable loading control, with consistently equal band intensities observed across all lanes in all four Western blots, validating uniform protein loading. The Western blot images and corresponding densitometric graphs for all four proteins are presented (Figure 8).

## Effects of Cephalotaxine on RUNX2 Protein Expression in Human Osteosarcoma Cells (MG-63 Cells)

The effects of Cephalotaxine on RUNX2 protein expression which is the master transcription factor governing osteoblastic differentiation and a critical molecular regulator of osteosarcoma cell invasion and metastasis was evaluated by Western blot analysis in MG-63 human osteosarcoma cells. Representative Western blot images and corresponding densitometric quantification graph are presented in Figure 8. As demonstrated in Figure 8, RUNX2 protein expression was significantly upregulated in Cephalotaxine-treated MG-63 cells in a clear and progressive dose-dependent manner relative to the untreated control group. In the Western blot images, the untreated control cells displayed a comparatively faint and low-intensity RUNX2 band. Treatment with Cephalotaxine at 5 µM produced a visibly more intense

RUNX2 band compared to the untreated control lane, while the 10 µM Cephalotaxine-treated lane exhibited the strongest and most intensely stained RUNX2 band among all three lanes, reflecting the highest RUNX2 protein abundance at this concentration. Beta-actin bands were consistently and uniformly equal in intensity across all three lanes, confirming equal protein loading. The significant dose-dependent upregulation of RUNX2 protein expression by Cephalotaxine at both concentrations directly corroborates and validates the corresponding upregulation of RUNX2 mRNA expression demonstrated by qRT-PCR analysis, collectively establishing robust dual transcriptional and translational evidence for Cephalotaxine-mediated activation of RUNX2-driven osteoblastic differentiation signalling in MG-63 osteosarcoma cells.

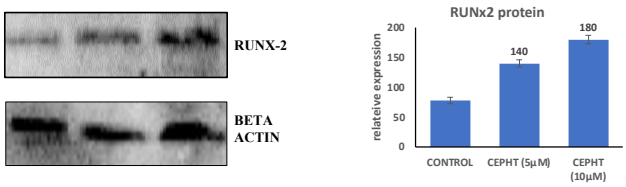


**Figure 8.** Effects of Cephalotaxine on RUNX2 protein expression of osteogenic differentiation markers in MG-63 human osteosarcoma cells as determined by Western blot analysis. Western blot images (left panel) and corresponding densitometric quantification graph (right panel) showing relative protein expression of RUNX2 in untreated control and Cephalotaxine-treated MG-63 cells (CEPHT 5 µM and CEPHT 10 µM). Beta-actin (β-actin) was used as the internal loading control to normalise protein expression across all lanes. Data are expressed as mean ± SD from three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test.

**Effects of Cephalotaxine on ALP Protein Expression in Human Osteosarcoma Cells (MG-63 Cells)**

The effect of Cephalotaxine on the protein expression of Alkaline Phosphatase (ALP) which is a membrane-bound glycoprotein enzyme serves as a early-to-mid stage marker of osteoblastic differentiation whose enzymatic activity is essential for matrix mineralization. Representative Western blot images and densitometric quantification are presented in Figure 9. As shown in Figure 9, ALP protein expression was significantly and dose-dependently upregulated in Cephalotaxine-treated MG-63 cells compared to the untreated control group. Western blot analysis revealed a faint and low-intensity ALP band in the untreated control lane. Cephalotaxine treatment at 5 µM produced a visibly more intense ALP protein band compared to the control lane, while treatment at 10 µM produced the most intensely stained and thickest ALP band among all three lanes, clearly reflecting the highest ALP protein abundance at this concentration. The significant dose-dependent upregulation of ALP protein expression is consistent with and directly corroborated by the corresponding upregulation of ALP mRNA demonstrated by qRT-PCR and the enhanced ALP enzymatic activity confirmed by histochemical ALP staining, collectively establishing three-level transcriptional, translational, and functional evidence for Cephalotaxine-induced osteoblastic differentiation in MG-63 osteosarcoma cells.

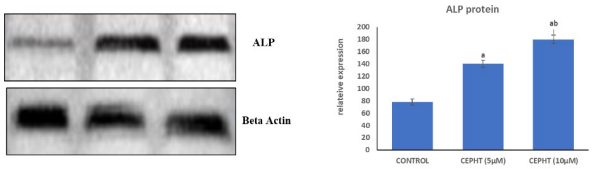


**Figure 9.** Effects of Cephalotaxine on ALP protein expression of osteogenic differentiation markers in MG-63 human osteosarcoma cells as determined by Western blot analysis. Western blot images (left panel) and corresponding densitometric quantification graph (right panel) showing relative protein expression of ALP in untreated control and Cephalotaxine-treated MG-63 cells (CEPHT 5 µM and CEPHT 10 µM).

**Effects of Cephalotaxine on BMP-2 Protein Expression in Human Osteosarcoma Cells (MG-63 Cells)**

The effect of Cephalotaxine on the protein expression of BMP-2 (Bone Morphogenetic Protein-2) which is a potent osteoinductive growth factor was assessed by Western blot analysis in MG-63 human osteosarcoma cells. Representative Western blot images and corresponding densitometric quantification are presented in Figure 10. As illustrated in Figure 10, BMP-2 protein expression was significantly upregulated in Cephalotaxine-treated MG-63 cells in a dose-dependent manner compared to the untreated control. Treatment with Cephalotaxine at 5 µM produced a visibly more intense BMP-2 band compared to the control lane, while treatment at 10 µM yielded the strongest and most intensely stained BMP-2 band among all three lanes, reflecting maximum BMP-2 protein upregulation at this concentration. Beta-actin bands showed consistently equal intensity across all three lanes, confirming equal protein loading and validating the BMP-2 expression differences as genuine treatment effects. Densitometric quantification normalized to β-actin confirmed the dose-dependent upregulation of BMP-2 protein expression.

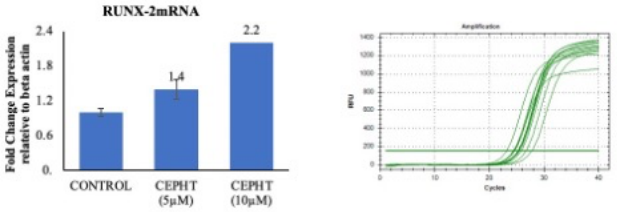


**Figure 10.** Effects of Cephalotaxine on BMP-2 protein expression of osteogenic differentiation markers in MG-63 human osteosarcoma cells as determined by Western blot analysis. Western blot images (left panel) and corresponding densitometric quantification graph (right panel) showing relative protein expression of BMP-2 in untreated control and Cephalotaxine-treated MG-63 cells (CEPHT 5 μM and CEPHT 10 μM).

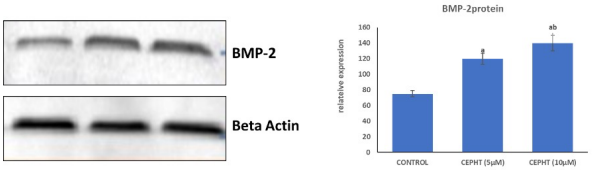


Figure 11. Effects of Cephalotaxine on Osteocalcin protein expression of osteogenic differentiation markers in MG-63 human osteosarcoma cells as determined by Western blot analysis. Western blot images (left panel) and corresponding densitometric quantification graph (right panel) showing relative protein expression of Osteocalcin in untreated control and Cephalotaxine-treated MG-63 cells (CEPHT 5 μM and CEPHT 10 μM).

### Effects of Cephalotaxine on Osteocalcin Protein Expression in Human Osteosarcoma Cells (MG-63 Cells)

The effect of Cephalotaxine on the protein expression of Osteocalcin (OCN) which is the most abundant non-collagenous bone matrix protein synthesised and secreted by fully mature and terminally differentiated osteoblasts, representing the most specific and definitive late-stage protein marker of completed osteoblastic differentiation and bone matrix formation was evaluated by Western blot analysis in MG-63 human osteosarcoma cells. Representative Western blot images and densitometric quantification graphs are presented in Figure 11. As demonstrated in Figure 11, Osteocalcin protein expression was significantly upregulated in Cephalotaxine-treated MG-63 cells in a dose-dependent manner relative to the untreated control group. Among all four proteins evaluated by Western blot in this study, Osteocalcin demonstrated the lowest baseline expression in untreated control cells, with a faint and barely detectable band in the control lane, consistent with the characteristically low baseline Osteocalcin protein expression in undifferentiated MG-63 osteosarcoma cells. Treatment with Cephalotaxine at 5 μM produced a visibly more intense Osteocalcin band compared to the faint control lane, reflecting significant induction of Osteocalcin protein expression even at the lower treatment concentration. At 10 μM Cephalotaxine, the strongest and most intensely stained Osteocalcin band among all three lanes was observed, reflecting maximum Osteocalcin protein upregulation at this concentration. Beta-actin bands showed consistently equal and uniform intensity across all three lanes, confirming equal protein loading and validating the observed Osteocalcin expression differences as authentic treatment effects. The significant upregulation of Osteocalcin protein, the most specific marker of mature osteoblastic differentiation, directly corroborates the corresponding upregulation of Osteocalcin mRNA demonstrated by qRT-PCR, collectively establishing conclusive dual transcriptional and translational evidence for Cephalotaxine-induced terminal osteoblastic differentiation in MG-63 human osteosarcoma cells.

## DISCUSSION

Osteosarcoma is identified by the uncontrolled proliferation of malignant osteoblasts, along with dysregulated osteogenic signalling and abnormal mineralisation of the bone matrix. Emerging data indicates that focusing on osteogenic transcription factors and growth-factor–mediated survival pathways may constitute an effective therapeutic approach for the management of osteosarcoma. The current study demonstrated the anti-osteosarcoma efficacy of the natural alkaloid Cephalotaxine in MG-63 human osteosarcoma cells by utilising a combination of methodologies. Cephalotaxine, an alkaloid derived from Cephalotaxus species, has surfaced as a structurally distinctive alternative, however its molecular targets in osteosarcoma are inadequately defined. Even with improvements in surgical resection and multimodal chemotherapy regimens, the five-year survival rate for metastatic osteosarcoma remains around 30%, highlighting the critical clinical need for therapeutics with different mechanisms of action. Natural alkaloids have garnered significant interest as sources of bioactive scaffolds, several of which have shown strong anticancer effects with advantageous selectivity profiles.

Cephalotaxine, an alkaloid derived from Cephalotaxus species, has surfaced as a structurally distinctive alternative, however its molecular targets in osteosarcoma are inadequately defined. The current study aimed to systematically assess the cytotoxic, anti-proliferative, and pro-differentiative effects of Cephalotaxine in human osteosarcoma MG-63 cells, with a specific focus on its influence on cell viability, mRNA and protein expression of vital osteogenic and bone-resorption markers, and the mineralisation of the extracellular matrix. The

experimental findings, in connection with the current research, provide a mechanistic foundation for the observed therapeutic advantages. The MTT analysis revealed that after treatment with cephalotaxine which drastically lowered the viability of MG-63 cells in a way that depended on the concentration. The gradual decrease in metabolic activity at elevated doses (10–20 μM) indicates the significant antiproliferative impact of cephalotaxine on osteosarcoma cells. These findings demonstrate that cephalotaxine disrupts cellular survival signalling mechanisms that control tumour development. Prior research has shown that osteosarcoma proliferation is significantly regulated by insulin-like growth factor signalling, especially through IGF-1R activation, which enhances tumour cell viability and metastasis.

Cephalotaxine's activation of the mitochondrial apoptosis pathway caused leukaemia cells to undergo apoptosis. In addition, Cephalotaxine stopped the acidification of lysosomes and disrupted the flow of autophagy. ceasing the flow of autophagy accelerated cell death and made it more challenging to get rid of damaged mitochondria **[14]**. Real-time PCR analysis revealed the significant modulation of osteogenic and bone remodelling markers following cephalotaxine treatment. Specifically, the expression levels of RUNX2, ALP, osteocalcin (OCN), and RANKL were markedly reduced in treated MG-63 cells, while OPG expression exhibited regulatory changes. RUNX2 is a master transcription factor controlling osteoblast differentiation and is frequently over-expressed in osteosarcoma, contributing to tumor progression and abnormal bone formation. The downregulation of RUNX2 observed in this study indicates that cephalotaxine may suppress osteosarcoma development by interfering with osteogenic transcriptional programs. Similarly, reduced expression of ALP and osteocalcin, which are classical markers of osteoblast maturation and matrix mineralization, suggests inhibition of pathological osteogenic differentiation in malignant osteoblasts. Furthermore, modulation of the RANKL/OPG axis indicates a potential regulatory role of cephalotaxine in bone remodelling pathways. The RANKL signalling pathway is known to promote osteoclast activation and bone destruction in osteosarcoma microenvironment. Therefore, regulation of this axis may contribute to restoring the balance between bone formation and resorption.

A CET-type alkaloid, Isoharringtonine, inhibited STAT3 signalling and breast cancer stem-like characteristics **[15]**. The paclitaxel side chain and its isomers added acyl groups to the C3 hydroxy groups of Cephalotaxine and drupacine, which led to the synthesis of many derivatives **[16]**. The proliferation of KB (Oral squamous cell carcinoma), HCT (Colorectal carcinoma), and Bel (Erythroid and Liver cancer) cell lines has been significantly inhibited by compounds VIIIa, VIIIb, IXa, and IXc. Additionally, cephalotaxine was found to suppress the growth of HepG2 cells in a manner that was both dose- and time-dependent **[17]**. It also improved the organelles of acid vesicles. The autophagy markers LC3-II and Beclin-1 were also activated by CET. C-3-epi-wilsonione, an alkaloid derived from homoerythrina, demonstrated the cytotoxicity against a considerable number of human cancer cell lines **[18]**. Western blot analysis demonstrated a consistent, dose-dependent upregulation of RUNX2, ALP, BMP-2, and osteocalcin proteins in Cephalotaxine-treated MG-63 cells, corroborating the RT-qPCR findings at the translational level. RUNX2 protein expression showed a statistically significant increase. Similarly, ALP and BMP-2 proteins were upregulated in a comparable dose-dependent pattern, and osteocalcin protein abundance increased substantially at the higher concentration.

Homoharringtonine [HHT] on osteosarcoma, inhibited cell migration and there is no disruption of microtubules was observed in In vitro studies **[19,20]**. Matrix mineralization plays an important role in osteosarcoma because malignant osteoblasts produce abnormal mineralized osteoid. In the present study, Alizarin Red S staining demonstrated a marked reduction in calcium deposition and mineralized nodule formation on cephalotaxine-treated MG-63 cells. Quantitative analysis further revealed a concentration-dependent decrease in mineralization. These findings suggest that cephalotaxine suppresses osteogenic activity in osteosarcoma cells. The observed inhibition of mineralization is consistent with the reduced expression of RUNX2, ALP, and osteocalcin genes, which are essential regulators of osteoblast differentiation and matrix maturation. The majority of research has shown how important ALP are for mineralising bones. ALP is the main gene that starts the process of calcifying bone, bone tissue, and cartilage **[21]**. Retinoic acid, parathyroid hormone, and vitamin D are some of the drugs which promote the production of ALP, which helps bones calcify **[22-24]**. During treatment with the phytochemical citral, there is a reduction in ALP expression was observed which may be due to upregulation of osteocalcin since ALP is an osteogenic factor responsible for the initial phase of mineralization and it also decreased with time duration. Citral induced osteoblast differentiation through stimulating the osteogenic stimulator ALP in human osteoblast-like MG-63 cells confirms the considerable amplification in the activity of ALP **[25]**.

Cephalotaxine's multi-target pharmacological nature, encompassing antiproliferative, pro-differentiative, anti-resorptive, anti-mineralogenic, and anti-metastatic activities, makes it an excellent option for further

preclinical development against osteosarcoma. Further studies are required to explore Cephalotaxine's performance in 3D tumour spheroid models, analyse its impact on osteosarcoma migration and invasion evaluations, elucidate its pharmacokinetic profile in vivo, and investigate its combination effectiveness with conventional osteosarcoma chemotherapeutic drugs, like doxorubicin and cisplatin.

## CONCLUSION

Our current research presents strong experimental evidence that the natural alkaloid Cephalotaxine has strong, multifaceted anti-osteosarcoma effects in human MG-63 cells, working through molecular mechanisms that are different yet work together. The important findings are Cytotoxicity, Osteogenic Gene Upregulation by Real-Time PCR analysis, Protein Expression Validation by Western blot analysis and finally Matrix Mineralization Suppression by Alizarin Red S and ALP staining assays demonstrated the multifaceted anti-osteosarcoma effects in human MG-63 cells. Overall, these results show that Cephalotaxine is a natural alkaloid with a wide range of mechanisms of action and a lot of potential as a treatment for osteosarcoma. More research is needed to see how it functions through in vivo xenograft models, caspase activation assays, Smad pathway analysis, and pharmacokinetic profiling for clinical translation.

## CONFLICT OF INTEREST

There is no conflict of interest as expressed by the authors.

## ACKNOWLEDGEMENT

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