THE EFFECT OF GLUCURONIDATION ON CURCUMIN BIOACTIVITY IN BONE METASTATIC BREAST CANCER

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ABSTRACT

Breast cancer bone metastasis is characterized by the formation of osteolytic lesions via a TGFβ-dependent vicious cycle. Although there is currently no cure for these lesions, previous studies show that turmeric-derived curcuminoids (CURC) may inhibit this pathway in estrogen receptor negative (ER–) breast cancers. However, most circulating CURC exists in its glucuronidated form (G-CURC), so this study sought to determine the effects of glucuronidation on CURC bioactivity in multiple ER– breast cancer cell lines. The results reveal that CURC decreases phosphorylation of Smad proteins, a key step in the TGFβ signaling pathway, in multiple ER– breast cancer cell lines, while G-CURC has no effect. Furthermore, secretion of TGFβ-induced parathyroid hormone-related protein (PTHrP), a key osteolytic factor in bone metastasis, was inhibited in ER– cells lines following CURC treatment and subsequent TGFβ stimulation. These data suggest that aglycone CURC inhibits the Smad-dependent TGFβ signaling pathway involved in the breast cancer bone metastases and may prevent release of osteolytic factors that aid in bone resorption, while G-CURC is not biologically active. Since G-CURC is the predominate form in circulation, future studies are necessary to characterize the means through which G-CURC may be converted to CURC, systematically and/or within the bone microenvironment.
BACKGROUND

Breast cancer is one of the leading causes of cancer deaths among women in the United States, second only to lung cancer. In 2015, there were approximately 292,000 reported cases of breast cancer, resulting in over 40,000 deaths. Breast cancer, like all types of cancer, is characterized by uncontrolled and/or abnormal cell growth. The American Cancer Society identifies two main types of breast cancer—in situ and invasive, with invasive being more common. In both types, abnormal cells tend to arise in the lobules or ducts of the breast, which are responsible for milk production. Compared to in situ, invasive breast cancers spread to surrounding tissue and can metastasize to distance sites. In addition to being more common, invasive breast cancers are also more difficult to treat and associated with a poorer prognosis.

It is important to note that invasive breast cancer may be classified as one of four main molecular subtypes—luminal A, luminal B, HER2-enriched, and triple negative. These are categorized based on the absence or presence of estrogen/progesterone hormone receptors (HR), and whether or not there are elevated levels of the human epithelial derived growth factor receptor 2 (HER2). Triple negative breast cancers, which lack receptors for estrogen and progesterone and do not express excess HER2, only make up about 12% of breast cancer cases but are associated with a poor prognosis. Additionally, there are currently no treatments that specifically target triple negative breast cancers. Other classifications of breast cancers exist, but triple negative breast cancers are relevant to the contents of this study.

As previously mentioned, breast cancer tends to metastasize, or spread, to other areas of the body, and in most cases, metastases at distant organs are the cause of mortality. Common areas of metastasis include lung, liver, and bone, the latter of which will be the focus of this paper. Breast cancer bone metastasis presents in two main forms: osteoblastic or osteolytic.
lesions. Osteoblastic lesions are characterized by the formation of new bone, while osteolytic lesions involve the resorption of existing bone. Osteolytic lesions are far more common, with close to 90% of patients with advanced staged breast cancer having one or more of these lesions. Not only can these bone lesions cause severe pain, pathologic fractures, hypercalcemia and nerve compression syndromes, but they are essentially incurable. In other words, once breast cancer has spread to bone, palliative care, rather than curative, becomes the primary approach.

Although the type of lesion resulting from metastasis is somewhat variable, it is clear that breast cancer cells have a tendency to spread to bone. The mechanism for this process is still not entirely elucidated, although several ideas have been proposed. The “seed and soil” hypothesis, which was proposed by Stephen Paget, suggests that the bone microenvironment may provide a nourishing "soil" that encourages the growth of breast cancer cells, the “seeds”. In order to better understand the mechanism through which breast cancer metastasis causes osteolysis, it is essential to understand what is meant by the “bone microenvironment”.

Bones are composed of two main structures—the hard-mineralized bone matrix and the bone marrow that fills the cavity of the bone. The bone matrix can also be further classified into the outer, shell-like “cortex” and the inner lattice of “trabecular” or “spongy” bone, the latter of which tends to be concentrated at the ends of long bones. The matrix of bone is home to a variety of growth factors, including, but not limited to, insulin-like growth factors (IGFs), transforming growth factor α and β (TGF-α and TGF-β), platelet-derived growth factors (PDGFs), and bone morphogenic proteins (BMPs). Sitting on the surface of the bone matrix are osteoclasts and osteoblasts, the major cells involved in maintenance of the bone. Osteoclasts resorb bone by degrading the bone matrix, while osteoblasts build new bone. Both of these cells are derived
from precursors found in the inner bone marrow, which is the primary site of hematopoiesis. Osteoclasts, which are derived from hematopoietic cells, are only formed in situ when needed (via cross talk with osteoblasts) in order to degrade bone, while osteoblasts, which are derived from bone stromal cells, are always present although not necessarily active. Normally, the osteoclasts and osteoblasts work in coordination to maintain adequate amounts of healthy bone throughout a person’s lifetime. However, this process appears to be disrupted following the arrival of metastatic tumor cells.

There is a great deal of evidence that suggests that breast cancer cells have the ability to stimulate osteoclast formation (osteoclastogenesis) by inducing hematopoietic stem cells to differentiate into osteoclasts. When osteoclasts degrade bone, TGFβ is released from the matrix and induces the tumor cell to produce osteolytic factors, including parathyroid hormone-related protein (PTHrP) and interleukins (IL-1, IL-8, IL-11, IL-15 and IL-17). PTHrP then stimulates the production of receptor activator of NF-κB ligand (RANKL) by osteoblasts, which binds to its receptor on osteoclasts precursors and further stimulates osteoclastogenesis. This induces a so-called vicious cycle that exacerbates the formation of osteolytic lesions, as seen in Figure 1.
As shown above, TGFβ plays a pivotal role in the vicious cycle that is thought to be responsible for the formation of these osteolytic lesions. TGFβ is a versatile cytokine that is normally expressed in developing and adult tissues, but it may be associated with several diseases, including cancer, when regulation is lost\(^6\). In healthy mammary epithelial cells, TGFβ negatively regulates growth, but in advanced cancers, it can act as a tumor promoter\(^3\). TGFβ signaling occurs through a variety of pathways, but this paper will focus on Smad-mediated TGFβ signaling. Active TGFβ ligand binds TGFβ-receptor type II, which recruits and
phosphorylates TGFβ-receptor type I via its intrinsic kinase activity. This allows TGFβ-receptor type I to phosphorylate Smad2 and/or Smad3. Phosphorylated Smad proteins form a multimeric complex with mediator Smad4, and this complex can then translocate to the nucleus and affect gene transcription (refer to Figure 2). In the context of bone metastatic breast cancer, translocation of the Smad complex to the nucleus results in upregulation of osteolytic factors, including PTHrP.

![Figure 2. Overview of Smad-mediated TGFβ signaling pathway. TGFβ binds TGFβ receptor II, resulting in phosphorylation of Smad proteins and downstream effects on gene transcription.](image)

Current treatments for these osteolytic lesions include bisphosphonates and denosumab, which directly target osteoclasts, preventing them from degrading bone either by inducing apoptosis or blocking differentiation, respectively. However, ongoing research is investigating potential alternative treatments that may block the vicious cycle by targeting tumor cell secretion...
of factors that drive osteolysis, an often-disregarded component of this vicious cycle. One such potential treatment are curcuminoids derived from rhizomes of the spice turmeric\textsuperscript{7,8}. Research suggests that curcumin, the primary turmeric-derived curcuminoid, may induce apoptosis in various cancer cell lines, and several \textit{in vitro} studies demonstrated that curcumin inhibits the growth of breast cancer cells\textsuperscript{7,8}.

With this information in mind, a more recent study investigated how curcuminoids may also specifically affect the TGF\(\beta\) signaling pathway involved in the vicious cycle of osteolytic lesion formation. Various \textit{in vitro} studies were performed using an estrogen receptor negative (ER-) human breast cancer cell line (MDA-MB-231, referred to here as MDA-SA) that is known to form osteolytic lesions dependent on TGF\(\beta\) stimulation of PTHrP secretion in a mouse model of breast cancer bone metastases. Curcuminoid treatment inhibited both TGF\(\beta\)-dependent Smad phosphorylation, as well as downstream release of TGF\(\beta\)-stimulated PTHrP from MDA-SA cells.\textsuperscript{5} Furthermore, curcuminoid administration inhibited the formation of osteolytic lesions in athymic nude mice inoculated with MDA-SA cells\textsuperscript{5}. Taken together, these data suggest that curcuminoids may have an inhibitory effect on the formation of osteolytic lesions caused by breast cancer metastasis through downregulation of tumor-cell TGF\(\beta\) signaling.

Although these findings are promising, it is essential to determine the efficacy of curcumin, as nearly 40\% of breast cancer survivors use herbal treatments, most of which are oral supplements\textsuperscript{5}. This brings up the important issue of bioavailability. \textit{In vivo} studies have shown that oral treatment with curcumin results in poor bioavailability as a result of curcumin metabolism. Following oral ingestion, curcumin is conjugated by the liver to curcumin-glucuronide, which is the major circulating metabolite\textsuperscript{9}. Glucuronidation is an enzymatic process that protects organisms from potentially toxic foreign substances. The addition of a glucuronide
moiety increases the polarity and solubility of the substrate, allowing for excretion via bile and/or urine\textsuperscript{10}. Although curcumin-glucuronide is thought to be the biologically inactive, it is interesting to note that there are cases in which glucuronidation results in a more active compound. A notable example is morphine, which becomes several times more active following conjugation to morphine-6-glucuronide\textsuperscript{10}. As glucuronidated-curcumin appears to be the primary metabolite in circulation, it is essential to determine which form is responsible for the observed \textit{in vivo} effects.

The aim of this study was two-fold. First, we wanted to determine the effect of glucuronidation on curcumin bioactivity in the context of bone metastastic breast cancer. This will allow us to determine whether aglycone (free) curcumin or glucuronidated-curcumin is responsible for inhibiting Smad-dependent, TGF\(\beta\) signaling. We also sought to confirm that the effects of curcuminoids on the TGF-\(\beta\) signaling pathway were consistent across multiple bone-tropic, ER-negative cell lines. MDA-SA cells will be tested once again and compared with three other triple negative cell lines that are known to form TGF\(\beta\)-responsive osteolytic bone metastases when inoculated into mice—MDA-1833 and MDA-2287, human cell lines that must be inoculated into immunocompromised mice (athymic nude), and 4T1, a murine cell line that can be inoculated into immunocompetent mice (BALB/c). We predict that free curcuminoids, but not glucuronidated-curcumin, will be biologically active in the context of TGF\(\beta\) signaling inhibition, and that this effect will be consistent across all four ER-, bone-tropic breast cancer cell lines.
METHODS

Cell culture

Four ER negative breast cancer cell lines were utilized throughout this study to compare the effects of curcumin on the TGF-β signaling pathway. MDA-MB-231 (MDA-SA) is a well characterized human breast cancer cell line that is known to form TGFβ-dependent osteolytic lesions\(^\text{11}\). For comparison, we chose two other human cell lines, MDA-1833 and MDA-2287, which are derived from MDA-SA, and one murine cell line, 4T1\(^\text{12,13}\).

All cell lines were maintained in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin (antibiotics) to allow for optimal growth conditions. Cells were grown in vented T-75 flasks and stored in an incubator at 37 degrees Celsius and 5% \text{CO}_2. Any cell work was performed in a laminar flow hood to avoid contamination.

Between experiments, cell cultures were split as necessary to avoid over-confluency and crowding of cells. To split cells, media was removed using a vacuum, and cells were rinsed once with 5 mL phosphate buffered saline (PBS) per T75 flask. After removing the PBS, 1 mL of trypsin was added per flask to allow cells to detach, followed by the addition of media to neutralize the trypsin. An appropriate amount of the cell solution was then added to a new T75 flask with fresh media depending on the intended use for the cells and the typical rate of growth, which varies among these cell lines.

MTT assay to determine cell viability

Prior to cell treatment with curcuminoids or glucuronidated-curcumin, a dose-response MTT cell proliferation assay was performed to determine cytotoxicity and optimal dosing. The
MTT assay is a colorimetric assay that measures metabolic activity and therefore is indicative of cell viability.

MDA-SA, MDA-1833, MDA-2287, and 4T1 cell lines were grown to sub-confluency in T-75 flasks, and then plated in 96-well plates (one plate per cell line) at 1x10⁴ cells/well. A hemocytometer was used to ensure that the correct amount of cells was plated, and Trypan blue allowed for the identification of dead cells. After plating, cells were allowed to adhere to the wells overnight. The following day, immediately prior to treatment, curcuminoids (Fisher AC218580100, 68% curcumin, 15.8% demethoxycurcumin, 4.9% bis-demethoxycurcumin) were serially diluted to achieve the following concentrations of curcuminoids: 1 µM, 3 µM, 10 µM, 18 µM, 30 µM, 56 µM, and 100 µM. Additional wells were plated with cells but did not receive treatment in order to serve as controls.

In addition, cells were treated with 100 µM glucuronidated-curcumin (Toronto Research Chemicals, Inc.) to match the highest dose of curcuminoids and therefore compare its effects on cell viability. Stock glucuronidated-curcumin (10 mg/mL, reconstituted in DMSO) was diluted into media to achieve a final concentration of 100 µM, matching the highest concentration of curcuminoids tested. All wells were refreshed with media with or without cucuminoids or glucuronidated-curcumin.

After 16 hours of curcuminoids or glucuronidated-curcumin exposure, treatments/media were aspirated and replaced with traditional media + 10% MTT reagent was added to all wells. An additional control of only MTT media (no cells) was used to subtract basal absorbance. After allowing cells to incubate with the MTT media for about three hours, purple precipitate formed, indicating that the MTT reagent has been reduced by mitochondria in living cells and the reaction has gone to completion. At this point, 100 µL of detergent was added to each well to
quench the reaction and solubilize the purple substrate. Plates were left in the dark at room temperature overnight, and the following day, a plate reader was used to record absorbance at 570 nm.

**Curcumin & glucuronidated-curcumin treatment for Western blot analysis**

All cell lines were grown to sub-confluency in T75 flasks. Cells were rinsed with PBS following removal of old media, and then 1 mL of trypsin was added and cells were allowed to detach from flask before neutralizing with 4 mL of media. Six T25 flasks containing 3 mL of fresh media each received one 1 mL of the cell solution. These cells were allowed to adhere for at least one day, or until confluent. Due to varying growth rates, some cell lines (e.g. MDA-2287) required more time to reach confluency. The following day, if confluent, control/curcumin/gluc-curcumin solutions were made up immediately prior to treatment.

First, stock curcuminoids (105 mg/mL in DMSO) was diluted at 1:10 in fresh media and subsequently diluted to give a final concentration of 30 µM (0.0111 mg/mL). Media with dimethyl sulfoxide (DMSO) in media was used as a control, and prepared in the same manner as curcuminoids. Stock glucuronidated-curcumin (10 mg/mL in DMSO) was also diluted into media, achieving a final concentration of 30 µM (0.0163 mg/mL). Old media was removed from each T25 and replaced with 4 mL of DMSO control, curcumin, or glucuronidated-curcumin containing media (2 flasks/treatment).

The following morning, TGFβ treatments were prepared. Stock TGFβ (20 µg/mL) was diluted into media at 1:100 to achieve a new concentration of 200 ng/mL. At the 16-hour mark post-treatment, 100 µL of TGFβ solution (200 ng/mL) was directly added to three of the six flasks (n=1 per treatment) and cells were allowed to incubate for one hour.
At the end of the incubation period, media/treatment solutions were removed from each flask and rinsed twice with PBS. A solution containing RIPA buffer (500 μL/flask) and phosphatase inhibitor (5 μL/flask) was added and flasks were incubated at 4°C for five minutes to allow for cell lysis. Following incubation, a cell scraper was used to rapidly scrape the flask in order to remove and lyse any residual cells. After transferring the lysate and removing a mucoid plug of nucleic acid, the lysate was centrifuged at 8000g for 10 minutes at 4°C to pellet and remove any cell debris. Supernatant was collected and aliquots were prepared for protein assay and future experiments. All protein samples were stored at -80°C.

**Western blotting**

Prior to beginning a western, protein assays were performed to ensure equal protein loading. Protein samples were diluted 1:100 with double distilled water. A standard curve was prepared using bovine serum albumin (BSA stock at 1.4 mg/mL). Both the protein samples and standard curves were run in duplicates on microtiter plates. Bradford dye reagent was added to all wells and absorbance was read at 595nm.

Samples for western blotting were prepared so that they contained the same amount of protein (based on concentrations obtained from protein assay), Laemmli Sample Buffer (Bio-Rad) with 5% β-mercaptoethanol, and RIPA buffer (Sigma). Samples were heated to ensure full protein denaturing, and then run on 15-well polyacrylamide, tris-glycine gels (Bio-Rad) at 200 volts. After samples were allowed to fully migrate through the gel, as evidenced by the ladder protein separation, the proteins were transferred from gels to PVDF membranes (0.45 μm pore).

Before antibody hybridization, all blots were blocked for one hour using TBS-T + 5% milk to prevent non-specific binding of the antibody. Primary antibodies, to either Smad2 (Cell
Signaling Technology [CST] #3108) or pSmad2 (CST #5339) were diluted into TBS-T + 5% BSA and allowed to incubate overnight at 4°C. The following day, HRP-conjugated secondary antibodies were diluted in TBS-T + 5% milk and allowed to incubate with the blots for one hour at room temperature. Blots were visualized using a chemiluminescence HRP-substrate solution (Thermo) and a UVP imager. Duration of exposure was based on strength of the signal.

**PTHRP Assay**

Of the four cell lines tested in this study, only MDA-SA and 4T1 secrete parathyroid hormone related protein (PTHRP), an osteolytic factor involved in the progression of bone metastatic breast cancer. To determine the effect of curcuminoids on secretion of PTHrP from these two cell lines, a commercial immunoradiometric assay (Diagnostic Systems Laboratory) was utilized. MDA-SA cells treated with 30 µM curcuminoids or control media for 4 hours and then stimulated with TGFβ for 24 hour, with four technical replicates per treatment.

**Data Analysis**

Prism 6.0 software was utilized to analyze data from both protein and MTT assays. Protein concentrations were determined using a standard curve on a semilog-scale, and half-maximal inhibitory concentrations were found using a dose-response also on a semilog scale. Densitometry analysis of western blots was performed using ImageJ software. All lanes were normalized to β-actin and phosphorylation of Smad2 was expressed as a ratio relative to constitutive Smad2 expression. For PTHrP data, differences between means were analyzed using one-way ANOVA, followed by Tukey’s post-hoc analysis. Significance was set at p<0.05.
RESULTS

Free curcuminoïds, but not glucuronidated-curcumin, exhibit cytotoxic effects

According to the MTT assay, curcumin displayed similar cytotoxic profiles across all cell lines. The curcuminoïd dose response curve (Figure 3a) revealed $IC_{50}$ of 42 µM, 41 µM, 32 µM, and 36 µM for MDA-SA, MDA-1833, MDA-2287, and 4T1, respectively, which were not statistically different between cell lines ($p < 0.2$). At low doses of curcuminoïds (18 µM or less), there was no statistically significant decrease in cell viability. Cell lines treated with 100 µM glucuronidated-curcumin appeared similar to control and did not result in significant cell death, while all cell lines treated with 100 µM curcuminoïds experienced less than 50% cell viability, as shown in Figure 3b. For all cell lines, 30 µM was chosen as the optimal treatment dose of both curcuminoïds and glucuronidated-curcumin, as it was the highest dose that did result in appreciable cell death.
Figure 3. (a) Dose-response curuminoid treatment to determine cytotoxicity in MDA-SA, MDA-1833, MDA-2287, and 4T1 cell lines. Cell viability is expressed as a percent of control (no curuminoid treatment). (b) Cell viability of curuminoids vs. glucuronidated-curcumin at 100 µM.
**Only free curcuminoids inhibits Smad-mediated TGFβ signaling**

All cell lines experienced some decrease in constitutive Smad2 expression as a result of curcuminoid treatment, regardless of whether or not cells were stimulated with TGFβ (Figure 4a-d). Constitutive Smad2 expression was not affected by glucuronidated-curcumin treatment. Curcuminoid treatment decreased TGFβ-stimulated Smad phosphorylation compared to control and glucuronidated-curcumin, which had no effect (Figure 5a-d).

In order to determine whether the observed effects were a result of decreased Smad phosphorylation and not simply a lack of available Smad proteins, effects of curcuminoid treatment on the ratio of pSmad2/Smad2 were determined. Densitometry analysis of the Western blots revealed a decrease in the ratio of pSmad2/Smad2 for all TGFβ-stimulated, curcuminoid-treated cell lines, compared to control or glucuronidated-curcumin, with the exception of MDA-2287 cells. While MDA-2287 cells did experience a decrease in Smad phosphorylation as a result of curcuminoid treatment and subsequent TGFβ stimulation, the decrease in pSmad2/Smad2 was minimal (20% decrease) suggesting that the decrease in pSmad2 in this cell line was primarily due to the curcuminoid-induced decrease in constitutive Smad2 levels. Treatment with 30 μM glucuronidated-curcumin for 16 hours, followed by one hour of stimulation with TGFβ, did not result in decreased Smad phosphorylation.

Interestingly, some cell lines (MDA-1833 and potentially MDA-2287), seem to express a small amount of phosphorylated Smad2 when treated with curcuminoids in the absence of TGFβ.
Figure 4. Curcuminoid, but not glucuronidated-curcumin, decreases TGFβ-stimulated pSmads2 levels and constitutive Smad2 levels. Western blots and corresponding densitometry analysis for (a) MDA-SA, (b) 4T1, (c) MDA-1833, and (d) MDA-2287. Cells were treated with DMSO-media control, 30 µM CURC, or 30 µM G-CURC for 16 hours, then stimulated with TGFβ for one hour. Figure 5. pSmad2 is expressed as a ratio to constitutive Smad2 expression.
Curcuminoids inhibit TGFβ-stimulated PTHrP secretion from MDA-SA and 4T1

Both MDA-SA and 4T1 secrete PTHrP when stimulated with TGFβ, although both constitutive and TGFβ-stimulated secretion differed between cell lines. 4T1 cells released close to 600 pg/mL when stimulated with TGFβ, while MDA-SA cells did not exceed 150 pg/mL. When MDA-SA cells were treated with curcuminoids and stimulated with TGFβ, PTHrP secretion returned to basal levels, a decrease of approximately four-fold.

4T1 cells experienced a statistically significant decrease (37%) in TGFβ-stimulated PTHrP as a result of curcuminoid treatment, although levels did not return to baseline.

Figure 6. PTHrP secretion from MDA-SA (6a-b) and 4T1 (6c-d) cell lines, in the presence or absence of curcuminoid treatment, with or without TGFβ stimulation. PTHrP secretion is expressed as both raw values (6a, 6c) and as a percentage of media control (6b, 6d). *p < 0.0001.
DISCUSSION

This study sought to determine the effect of glucuronidation on curcumin bioactivity in regards to bone metastatic breast cancer. As hypothesized, the results indicate that glucuronidated-curcumin is biologically inactive, while aglycone curcumin inhibits tumor-cell Smad-dependent TGFβ signaling and TGFβ-stimulated PTHrP secretion, an osteolytic factor that drives bone resorption.

These effects are not isolated to a single cell line but are consistent across multiple ER-bone-tropic breast cancer cell lines of both human and murine origin, although the degree of inhibition is somewhat variable. Curcuminoid treatment inhibited TGFβ-dependent Smad2 phosphorylation in all cell lines, with the effect occurring to a lesser degree in MDA-2287 compared to other cell lines. This does not exclude the possibility that curcumin could inhibit bone destructive TGFβ signaling in this cell line, as MDA-2287 breast cancer cells may depend on other signaling pathways, such as the non-canonical mitogen-activated protein kinase (MAPK) signaling pathway, which may not be affected by curcumin\textsuperscript{14}. As reported in the literature, there is evidence that the MAPK pathway may be implicated in the vicious cycle\textsuperscript{15}.

As stated earlier, only MDA-SA and 4T1 cells secrete PTHrP, and we found that curcuminoid treatment significantly decreased TGFβ-stimulated PTHrP release. Although he majority of breast cancer bone metastases are PTHrP positive, other tumor-derived factors are osteolytic\textsuperscript{16}. Therefore, future experiments are necessary investigate the release of osteolytic factors from MDA-1833 and MDA-2287 cell lines (e.g. interleukins), as these cell lines are not PTHrP-dependent.

Although we have shown that curcuminoids have a direct toxic effect on breast cancer cells \textit{in vitro}, previous studies have shown that administration of curcuminoids directly into an
orthotopic xenograft breast cancer tumor had no effect on primary tumor size. These findings augment the idea that the effects of curcuminoids on bone metastases in vivo are not mediated through cytotoxic effects but rather through crosstalk between the bone and the tumor. According to our findings, curcuminoids inhibit this vicious cycle by disrupting tumor-cell TGFβ signaling. If the aforementioned cycle is indeed responsible for the formation of these osteolytic lesions, these results indicate that curcuminoids have the potential to prevent the onset or worsening of this pathological condition.

The greatest limitation of this study is the lack of some key statistics due to small sample sizes (n=1 for Western/densitometry data) due to time constraints. We were not able to determine whether or not the decrease in Smad2 phosphorylation was statically significant, so these experiments will need to be repeated. It is also important to note that there are several other more common subtypes of breast cancers, such as estrogen receptor positive (ER+), that are not addressed in the context of this study. We primarily focused on the effects of curcuminoids on the ER- subtype, so further studies would be essential to understand how the other classes of breast cancer respond to curcuminoids.

Future studies in our lab intend to investigate the effects of curcuminoid treatment on phosphorylation of Smad3 proteins, which we also suspected will also be inhibited. Furthermore, since the majority of circulating curcumin exists as the glucuronide conjugate, a form that we have confirmed is biologically inactive, further research is necessary to determine if mechanisms exist that allow glucuronidated-curcumin to be converted to its biologically active form. Endogenous enzymes exist that are capable of removing the glucuronide conjugate to regenerate the original compound, so this is certainly an area for exploration, particularly within the bone microenvironment as hemopoietic cell are known to express deconjugating enzymes.
Numerous studies have already highlighted the anti-inflammatory, hypoglycemic, antioxidant, and antimicrobial properties of curcumin. More recently, curcumin has been identified as a potential chemotherapeutic agent in a multitude of cancers, including prostate, pancreatic, and colorectal cancer. Here we have identified that curcuminoids inhibit a key pathway involved in the pathogenesis of bone metastatic breast cancer, which appear to be consistent across various human cell lines, as well as one murine cell line. Although several questions remain unanswered regarding the role of curcuminoids, these findings encourage the necessity for further research to determine potential clinical applications.
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