Effect of Curcumin and Meriva on the Lung Metastasis of Murine Mammary Gland Adenocarcinoma

ABDELAZIM IBRAHIM1, ABDELMOUNEIM EL-MELIGY1, HAMDY FETAIH1, AMINA DESSOUKI1, GEORGE STOICA2 and ROLA BARHOUMI3

1Department of Pathology, College of Veterinary Medicine, Suez Canal University, Ismailia, Egypt; 2Department of Veterinary Pathobiology, and 3Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX 77843-4458, U.S.A.

Abstract. Background: Curcumin is one of the most studied natural compounds which has been used as a feed additive for centuries. Curcumin exhibits low oral bioavailability in rodents and human. Curcumin formulated with phosphatidylcholine (Meriva) increases curcumin bioavailability five-fold compared to original curcumin. The aim of this study was to evaluate the efficacy of curcumin conjugated with phosphatidylcholine as an anticancer agent. Materials and Methods: In this xenograft study, mammary gland tumor cell line (ENU1564) was inoculated into the mammary fat pad of athymic nude mice. The mice were treated orally with either curcumin or Meriva. The tumor and its lung metastasis were evaluated grossly, microscopically, and immunohistochemically. Results: Meriva significantly reduced the expression of MMP-9 and lung metastasis of our cell line used in this experimental model. Conclusion: Curcumin conjugated with phosphatidylcholine increased the efficacy of curcumin as an anticancer agent.

Incidence of breast cancer has increased throughout the years and most likely due to the development of new technologies for cancer detection. Breast carcinoma has a propensity for distant organ metastasis, and the lung and the pleura are among the most common metastatic sites (1). Until now there was no effective treatment available for metastatic breast cancer that follows surgery, radiation, and chemotherapy for the primary tumor (2). Recently, there has been a renewed interest, especially in developed countries, in using plants to treat livestock, pets, and human diseases, as they are less toxic, safer, and less expensive than manufactured drugs.

Matrix metalloproteinases (MMPs) are a family of Zn-dependent endopeptidases responsible for the degradation of extracellular matrix proteins. Activity of MMPs is controlled by both the activation of proenzymes and inhibition of tissue inhibitor of metalloproteinases (TIMPs) (3). MMP-2 and -9 (gelatinases), a group within the MMP family, have been shown to be capable of degrading of the critical basement membrane and extracellular matrix components, during the process of tumor invasion and metastasis (4).

Curcumin, a yellow colored polyphenol, is one of the most studied natural compounds and has been used as a feed additive for centuries. Curcumin, also known as (diferuloylmethane) is an active principle of the perennial herb Curcuma longa (commonly known as turmeric) (5). It has a wide range of beneficial properties, including anti-inflammatory, antioxidant, chemo-preventive and chemo-therapeutics activity (6). In vitro, Curcumin has been shown to inhibit the expression of MMP-9 in human astroglialoma cell lines (7). Some studies showed that curcumin has been the ability to reduce the metastasis of cancer in animal models of carcinogenesis through suppressing the expression of NF-κB and MMPs (8, 9). In a recent article, curcumin was shown to inhibit the metastasis of B16BL6, a highly metastatic melanoma cell line, in vitro and in vivo through down-regulating phosphatase of regenerating live-3 (PRL-3) expression (10). Other studies demonstrated that curcumin alone failed to affect the tumor growth and proliferation, but did have significant effect in combination with other compounds. These compounds either were chemical chemotherapy agents such as gemcitabine in the case of pancreatic cancer or natural component such as epigallo-catechin gallate, a flavonoid found in green tea (11, 12).
In phase I clinical trials, participants with cancer predisposition taking curcumin orally for 3 months showed little toxicity and revealed histological improvement in the precancerous lesion in 7 out of 25 patients (13). In addition, it has been shown that curcumin exhibits low oral bioavailability in rodents and may undergo intestinal metabolism; absorbed curcumin undergoes rapid first-pass metabolism and excretion in the bile (14).

In a rat experiment, Marczylo et al. (15) explored whether formulation with phosphatidylcholine increases the oral bioavailability or affects the metabolites profile of curcumin. Their results suggested that curcumin and its metabolites were five-fold higher in the plasma of rat following administration of curcumin formulated with phosphatidylcholine (Meriva) than the original one. In contrast, curcumin concentrations in the gastrointestinal mucosa after ingestion of curcumin formulated with phosphatidylcholine were somewhat lower than those observed in unformulated curcumin.

The aim of this study was to evaluate the use of curcumin and curcumin conjugated with phosphatidylcholine, in order to improve its bioavailability, as anticancer agents using a rat mammary adenocarcinoma cell line in a nude mouse xenograft model.

Materials and Methods

Chemicals. Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO, USA), whereas curcumin phospholipid complex (Meriva) was kindly provided by Indena SPA (Milan, Italy). The preparation of Meriva using EpiKuron 130 P, a de-oiled, powdered soybean lecithin enriched with 30% phosphatidylcholine is subject of an European patent application (EP 06004820), which was filed in March 2006. Meriva contained 16.89% curcuminoids, of which 93.82% was curcumin; the ratio of curcumin to Epikuron 130 P was 1:4 (15). Goat-antihuman MMP-9 polyclonal antibody and rabbit polyclonal anti-VEGF antibody were purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM), HANK’s balanced salt solution and fetal bovine serum (FBS) were purchased from Invitrogen Inc. (Carlsbad, CA, USA).

Cell lines. The ENU1564 tumor cell line used in this study was developed in our laboratory and originated from an N-ethyl-N-nitrosourea-induced mammary adenocarcinoma in a female Berlin-

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<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of animals</th>
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<tr>
<td>Control</td>
<td>Fed with Tekled 2019 powdered feed diet</td>
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<tr>
<td>Curcumin</td>
<td>Fed with Tekled 2019 containing 2% curcumin</td>
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<tr>
<td>Meriva</td>
<td>Fed with Tekled 2019 containing 6% Meriva</td>
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Druckrey IV (BD-IV) rat. This highly metastatic cell line (16) was maintained in DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were grown at 37°C in a humidified incubator containing 5% CO₂ in air. Cells were passaged biweekly and used for experiments in the exponential growth phase.

Cell viability assay. The cytotoxic effect of curcumin on ENU 1564 tumor cells was determined using 96® AQone assay kit (Promega, Madison, USA). The solution reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt (MTS) and an electron coupling reagent phenazole ethosulfate (PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The Cell Titer 96® AQone assay uses phenazole methosulfate (PMS) as the electron coupling reagent. Both MTS solution and MTS solution are supplied separately. Briefly, the cells were trypsinized, washed and then resuspended in DMEM medium and seeded into 96-well flat-bottomed plate (1×10⁴ cells/well) in 6 replicate. Curcumin was added to each well in various concentrations (0-40 μM). Cells were maintained in a humidified, 5% CO₂, 95% air incubator at 37°C for 24 hours or 48 hours. Following washing of cells, 100 μl of fresh DMEM and 20 μl of the Cell Titer 96® AQone Solution were added per well and incubated at 37°C for 1 hour and 30 min. All wells were sequentially read with a Biotek Synergy 4 plate reader (Biotek Instruments Inc., Winooski, VT, USA) using an absorbance wavelength of 490 nm. Mean background values were obtained by scanning blank wells. Results were expressed as mean optical density (O.D.) corrected to background.

Inoculation of ENU 1564 cells into nude mice. All experiments were performed according to the protocol that was reviewed and approved by the institutional Animal Care and Use Committee (SACC) at Texas A&M University. Forty-five-day-old female athymic nude mouse, Hsd Athymic Nude-Foxn1nu, were obtained from Harlan Laboratories (Indianapolis, USA). The animals were kept under a specific pathogen free environment with water and standard mouse chow ad libitum. The ENU 1564 cells were collected by trypsinization, washed and resuspended in HANK’s balanced salt solution. The mice were inoculated with 1×10⁶ ENU 1564 cells into the mammary fat pad. Inoculation was performed on animals under ketamine (87mg/kg, intramuscular injection)

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anesthesia. After inoculation, the animals were randomly divided into 3 groups (n=6), as indicated in Table 1. The Meriva content in our diet is 3-fold higher than that of curcumin in order to equalize the curcumin content in both diets. The animals were humanely euthanatized using pentobarbital (150 mg/kg, intraperitoneal injection) following ulceration of the tumor, and a complete necropsy was performed. The whole lung from each animal was cut into 7 sections, embedded into 2 paraffin blocks, and serial sections at different levels were prepared and stained with hematoxylin and eosin (H&E). The slides were then evaluated histologically and metastatic foci were counted.

Immunohistochemistry (IHC). Five-micron (5 μm) paraffin-embedded sections were prepared from formalin fixed tissues. The sections were deparaffinized in xylene and rehydrated with gradient alcohols. Antigen retrieval was performed by placing sections in citrate buffer (pH 6.0) and a decloaker pressure cooker for 15 minutes at 120°C per 18 psi. Following cool-down, potential non-specific binding sites were blocked with 5% normal goat or rabbit serum in phosphate-buffered saline (PBS). The sections were then incubated with primary cleave anti-MMP-9 (1:100) or anti-VEGF (1:100). After three 5-min washes in PBS, the sections were incubated with specific biotin-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA). A Vector-ABC streptavidin-peroxidase kit with a benzidine substrate was used for color development. Counter-staining was done with diluted hematoxylin. Sections that were not incubated with primary antibody served as negative control. Images were collected using Olympus vanox microscope and were analyzed using the “color range” tool available in Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) to extract the brown color for quantitation.

Statistics. Results for cell viability were analyzed and the half maximal inhibitory concentration (IC50) was determined using the sigmoidal dose response curve available in GraphPad Prism (GraphPad Software, San Diego, CA, USA). Comparison of data from two treatment groups was performed using a Student t-test and the difference was considered significant at p<0.05. Comparison of three treatment groups was performed using one way ANOVA, followed by Tukey multiple comparison test at p<0.05.

Results

Cytotoxicity of curcumin in ENU1564 cells. Prior to investigating the effect of Meriva and curcumin in vivo, the anti-proliferative and cytotoxic effects of curcumin on the ENU 1564 cells following 24 and 48 hours treatments were tested. Figure 1 shows that curcumin induced cytotoxicity in a time- and dose-dependent manner. The cell viability started to decrease at 25 μM following 24-hour treatment. The cytotoxicity of curcumin was more severe at 35 μM and 40 μM by 48 hours. The IC50 of curcumin after 24 and 48 hours was found to be 20 and 15 μM respectively (Figure 1). Under light microscopy, the cells treated with curcumin were limited in numbers, round, clumped in aggregates and detached from the surface of the wells (Figure 2B). Untreated ENU 1564 cells remained confluent (Figure 2A).

In vivo effect of curcumin and Meriva on mammary gland adenocarcinoma. To study and compare the effects of curcumin and Meriva on tumor growth and metastasis, the tumor was examined grossly and microscopically. The tumors were observed at the inoculation site on the 5th day post inoculation in all mice. The tumor grew rapidly and on the 17th day, the mice in the control group started to show ulceration of the skin over the tumor. On the 20th day, the Meriva group also began to exhibit ulceration. At the time of euthanasia, the tumors were large, ulcerated, and lobulated (Figure 3A). All of the control group animals and 3 out of 6 of the Meriva-treated group showed ulceration. On the cut surface, the tumors were whitish and contained yellowish soft areas that were interpreted as necrosis. Statistically, there was no significant difference between the groups regarding the tumor weight (Figure 3B). In some mice, the tumor penetrated the abdominal muscles and protruded onto the abdominal cavity. The axial lymph nodes were enlarged and showed minute white metastatic foci. The
In most of the animals, the lung showed multiple metastasis foci of variable size within the parenchyma and vascular channels (Figure 5A and Figure 5B). By counting the metastatic foci within the lung, it was evident that the Meriva treatment significantly decreased the number of metastatic foci in the lung ($p < 0.041$) in comparison to the control and curcumin groups (Figure 5C).

**Curcumin reduces the expression of MMP-9.** Increased concentration of MMPs and gelatinases in particular, are associated with invasion and metastasis (19). By examining the immunohistochemical staining for MMP-9 of tumor sections from the control and Meriva groups, a marked decrease of immunohistochemical staining of the MMP-9 was observed.
Figure 5. Sections of lung stained with H&E showing A: multiple metastatic foci invading the pulmonary tissue (×1, arrows) and B: metastatic tumor cells within the pulmonary arterioles (×10, arrow). C: Number of metastases in the lung originated from mammary tumors in control, curcumin- and Meriva-treated animals. Note that six animals were used per group and * indicates significant difference from control group at p<0.05.

Figure 6. MMP-9 immunohistochemistry of tumor sections A: control mice and B: Meriva-treated mice. C: Data represent levels of MMP-9 expression in both groups and * indicates significant difference between the two groups using the Student’s t-test at p<0.05.
Curcumin, a well-known dietary component from *Curcuma longa*, is an attractive candidate for drug development due to its ability to selectively kill tumor cells and not normal cells. Furthermore, *in vitro*, curcumin has been shown to behave as an antiproliferative and apoptotic agent in a wide variety of tumor cell lines and as an inhibitor of invasion, migration, and metastasis. The mechanisms involve modulation of multiple cell signaling pathways of the tumor cells, including cell proliferation pathways (cyclin D1, c-myc), cell survival pathways (Bcl-2, Bcl-xL, cFLIP, XIAP, c-IAP1), caspase activation pathways (caspase-8, 3, 9), tumor suppressor pathways (p53, p21), death receptor pathways (DR4, DR5), mitochondrial pathways, and protein kinase pathways (JNK, Akt, and AMPK) (21-32). Moreover, many studies have proved that curcumin can inhibit and treat a wide variety of cancer including prostate cancer, pancreatic cancer, gastric cancer, hepatocellular carcinoma, leukemia, and ovarian cancer in laboratory animals (33-35).

Unfortunately phase I clinical trials have shown that curcumin exhibits poor bioavailability as indicated by the curcumin level found in plasma and tissue. This is in general due to its poor absorption, rapid metabolism, and rapid systemic elimination. This means that high concentrations of curcumin cannot be achieved and maintained in plasma and tissues after oral ingestion. This is a major obstacle for the clinical use of this agent and suggests that the therapeutic potential of oral curcumin is limited (36-37). Multiple approaches are being sought to overcome these limitations. These include discovery of natural curcumin analogs from turmeric; synthesis of ‘man-made’ curcumin analogs; reformulation of curcumin with various oils and with inhibitors of metabolism (e.g. piperine); development of liposomal and nanoparticle formulations of curcumin; conjugation of curcumin prodrugs; and linking curcumin with polyethylene glycol. Conjugating curcumin with phospholipid leads to an increase in its bioavailability and could therefore induce better protection (38).

The aim of this study was to evaluate the effect of the curcumin phospholipid complex (Meriva) on a malignant and highly metastatic tumor cell line and to compare its effect to the parent compound, curcumin. Some studies have used curcumin phospholipid complex to treat diseases in animal models, such as carbon tetrachloride induced acute hepatic toxicity in rat (39) and to induce mammary epithelial cell apoptosis (40). To our knowledge, this is the first study which used Meriva to treat cancer *in vivo*.

The use of animal models to study tumor progression and metastatic behavior is an important tool for pharmacological evaluation of cancer therapy. Our cell line (ENU1564) is highly malignant and spontaneously metastasized to lung and lymph node following orthotopic inoculation into the mammary fat pad in rats and mice. ENU 1564 also provide a useful model of lung, brain, and bone metastatic breast cancer (16).

Before conducting the *in vivo* study, we investigated whether curcumin itself has a cytotoxic effect on ENU 1564 cells. As expected, our *in vitro* results showed that curcumin was very effective and significantly reduced the viability of the ENU 1564 cells with an IC50 of 20 μM within 24 hours and 15 μM within 48 hours, and this encouraged us to perform the *in vivo* study.

Our *in vivo* results revealed that both curcumin and Meriva had no significant effect on the tumor volume. This is in agreement with another study that showed that curcumin did not affect the incidence of animals with tumors in 7,12-dimethylbenz[a]anthracene-induced mammary cancer (DMBA) in Fisher 344 rat (41). In the same experiment, curcumin did not inhibit the colon adenocarcinoma induced in rat by azoxymethane (AOM).

Our next step was to determine if curcumin or Meriva reduced metastasis of the mammary gland adenocarcinoma to the lung. By counting the metastatic foci, our results showed that Meriva significantly inhibits the metastasis of the cells to the lung while curcumin had no significant effect on the metastasis. Our results are in disagreement with the results obtained by Bachmeier *et al.* (9) which showed that curcumin significantly inhibits the metastasis of human breast cancer cells to the lung. This observation may be specific to the tumor cells used in the study. Dissemination of metastatic cells probably occurs long before diagnosis of the primary tumor (9). However, the overall mortality for some of the common epithelial malignances, such as breast cancer, have been declining recently largely due to advances in early detection and prevention, the survival of patients with metastatic disease has not changed significantly over the past several decades (42).

MMPs play a major role in promoting tumor metastasis and overexpression of MMP-9 has been shown to be associated with the progression and invasion of tumors including mammary tumors (43, 44). Thus, the inhibitory effect on its expression is important for its usefulness as a therapeutic modality of cancer metastasis. MMP-9 protein expression was consistently significantly higher in neoplastic metastatic foci in the brain tissue of a syngeneic rat model of distant metastasis of ENU1564 (45). To confirm that Meriva decreases metastasis, we evaluated MMP-9 reactivity by IHC. Our results showed that Meriva significantly decreased the expression of MMP-9. It appears that curcumin or Meriva
does not alter the expression of VEGF in this animal model. This is consistent with the result that no difference in tumor growth and volume could be observed between the groups. Rapidly growing tumors require an activated angiogenesis process which correlated with increased expression of VEGF.

Western medicine has recently taken a great interest in curcumin and its anti-carcinogenic activities. Considerable research has been carried out and much more is needed in order to determine the mechanisms of action used by this popular compound. Due to its popularity and wide use throughout the world as a spice, a food preservative, and a coloring agent for foods and drugs, it could prove to be a valuable, inexpensive, and easily accessible chemotherapeutic agent for cancer. Overall, we can conclude from the above valuable, inexpensive, and easily accessible chemotherapeutic colorings agent for foods and drugs, it could prove to be a treatment for cancer metastasis in this animal model.

References


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