Curcumin Implants, Not Curcumin Diet, Inhibit Estrogen-Induced Mammary Carcinogenesis in ACI Rats

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Abstract
Curcumin is widely known for its antioxidant, anti-inflammatory, and antiproliferative activities in cell-culture studies. However, poor oral bioavailability limited its efficacy in animal and clinical studies. Recently, we developed polymeric curcumin implants that circumvent oral bioavailability issues, and tested their potential against 17β-estradiol (E2)-mediated mammary tumorigenesis. Female Augustus Copenhagen Irish (ACI) rats were administered curcumin either via diet (1,000 ppm) or via polymeric curcumin implants (two 2 cm; 200 mg each; 20% drug load) 4 days before grafting a subcutaneous E2 silastic implant (1.2 cm, 9 mg E2). Curcumin implants were changed after 4.5 months to provide higher curcumin dose at the appearance of palpable tumors. The animals were euthanized after 3 weeks, 3 months, and after the tumor incidence reached >80% (~6 months) in control animals. The curcumin administered via implants resulted in significant reduction in both the tumor multiplicity (2 ± 1 vs. 5 ± 3; P = 0.001) and tumor volume (184 ± 198 mm3 vs. 280 ± 141 mm3; P = 0.0283); the dietary curcumin, however, was ineffective. Dietary curcumin increased hepatic CYP1A and CYP1B1 activities without any effect on CYP3A4 activity, whereas curcumin implants increased both CYP1A and CYP3A4 activities but decreased CYP1B1 activity in the presence of E2. Because CYP1A and CYP3A4 metabolize most of the E2 to its noncarcinogenic 2-OH metabolite, and CYP1B1 produces potentially carcinogenic 4-OH metabolite, favorable modulation of these CYPs via systematically delivered curcumin could be one of the potential mechanisms. The analysis of plasma and liver by high-performance liquid chromatography showed substantially higher curcumin levels via implants versus the dietary route despite substantially higher dose administered. Cancer Prev Res; 7(4); 456–65. ©2014 AACR.

Introduction
Curcumin is a natural polyphenolic di-arylheptanoid obtained from rhizomes of Curcuma longa (turmeric) and has shown potent antioxidant, anti-inflammatory, and antiproliferative activities in various cell-culture studies (1, 2). Commercially, it is available as a mixture of 3 curcuminoids: curcumin I (diferuloylmethane, 75%), curcumin II (demethoxycurcumin, 20%), and curcumin III (bisdemethoxycurcumin, 5%; Supplementary Fig. S1; ref. 3). All the 3 curcuminoids have been shown to possess similar antiproliferative activities against leukemia, lung, prostate, pancreas and breast cancer cell lines (4), and cervical cancer cells (R. Munagala and R. Gupta; unpublished data). Curcumin’s ability to inhibit various enzymes (such as COX-2, CYP1B1, LOX), transcription factors (such as NF-κB and STAT3), and secondary messengers made it a highly desirable candidate to be developed as a drug. However, because of its poor solubility and high first pass metabolism, it showed limited oral efficacy in various preclinical and clinical studies (3, 5). As a result, various advanced drug delivery systems such as nanoparticles (6), liposomes (7), microparticles (8), micelles (9), hydrogels (10), micro-emulsions (11), and phospholipid mixtures (12) were developed to harness its full potential (3). However, because of rapid intestinal and hepatic metabolism, a drug delivery system of curcumin with continuous systemic administration is desired that can overcome the oral bioavailability issues.

Recently, we developed polymeric implants using poly (ε-caprolactone) (PCL) as the polymeric matrix to enable continuous administration of curcumin (for months to years) directly into the systemic circulation (13). Once grafted subcutaneously, these implants provide controlled release of curcumin continuously (24/7) at the implantation site from where it is systemically absorbed and distributed to various tissues (14). Biocompatibility and toxicity studies carried out using ACI rats revealed that these implants are safe and biocompatible with no apparent changes in physiological biochemistry (13). Hematologic parameters (such as WBCs, RBCs, platelets, basophils, eosinophils), and...
biochemical parameters of liver (AST, ALP, AP, amylase) and kidney (Na⁺, K⁺, Ca²⁺, creatinine, BUN) functions were also found to be unaffected by continuous systemic administration of massive doses of curcumin by these implants (13). Furthermore, these implants were found to maintain much higher plasma, liver, and brain concentrations for up to 3 months and required ~20-fold lower doses as compared with dietary administration (15).

Therefore, this study was designed to test chemopreventive efficacy of curcumin implants compared with curcumin delivered via diet against 17β-estradiol (E2)–induced mammary tumorigenesis in female ACI rats. Furthermore, mechanistic aspects for efficacy of curcumin implants were explored to deduce its mode of action against E2-induced mammary tumorigenesis.

Materials and Methods

Medical-grade PCL of 121,000 molecular weight (PCL-121) was purchased from SurModics Pharmaceuticals, and curcumin (C-3 complex extracted under GMP conditions for human use) was a generous gift from Sabinsa Corporation. Dichloromethane (DCM), ethoxyresorufin, and pyrene were from Sigma-Aldrich, polyethylene glycol of 8,000 molecular weight (PEG-8K) was from Fisher Scientific, and ethanol was from Pharmco-AAPER. Medical-grade silastic tubing (3.4 mm internal diameter) was purchased from Allied Biomedical.

Preparation of implants

Curcumin implants were prepared using solvent evaporation coupled with melt extrusion technique as described previously (13, 14). Briefly, PCL-121 and PEG-8K (65:35) were dissolved in DCM, whereas curcumin at a drug load of 20% (w/w) was dissolved in ethanol. The 2 solutions were mixed and the solvents were evaporated initially at 70°C, followed by drying at 65°C overnight under vacuum using savant SpeedVac (Thermo-Savant) to form a molecular dispersion of the drug in polymer. The molten polymer material was then extruded into silastic tubes (internal diameter 3.4 mm) at 65°C and cut into desired lengths to obtain cylindrical implants.

Animal studies

The animal studies were conducted after approval from the Institutional Animal Care and Use Committee of the University of Louisville. Female ACI rats (5- to 6-week old) purchased from Harlan Laboratories were acclimated to the facility for 1 week under vivarium conditions followed by the administration of AIN93M control diet. After 2 weeks, the animals were subdivided into 8 groups: (i) untreated control (n = 20), (ii) sham implants (n = 20), (iii) curcumin implants (n = 20), (iv) curcumin diet (n = 20), (v) E2 alone (n = 25), (vi) sham implants + E2 (n = 25), (vii) curcumin implants + E2 (n = 20), and (viii) curcumin diet + E2 (n = 20), for the 6-month time point. For the 3-week and 3-month time points, all the groups contained 6 animals each. A 1.2-cm silastic implant containing 9 mg E2 was grafted at the back of the rats as described previously (16). All the interventions—curcumin diet (1,000 ppm), curcumin implants (two 2-cm implants, 200 mg/implant, 20% w/w drug load), and sham implants—were grafted 4 days before E2 treatment and were continued until termination of the study. Animals were palpated weekly after 12 weeks of E2 treatment and were continued until termination of the study. Animals were euthanized by asphyxiation under CO₂ and all the animals were carefully examined for the presence of mammary tumors to calculate tumor volume and multiplicity as described above (16). Blood was collected by cardiac puncture. When the rats were bled on euthanasia, blood was divided into 2 tubes, one for serum and the other for plasma collected in a tube containing heparin. Serum from individual animals was used for E2 analysis. Plasma from individual animals was used for prolactin measurement. The remaining plasma samples were pooled to generate enough volume to measure curcumin levels for the 3-week and 3-month time points, and from individual animals (every third rat) for the 6-month time point. The liver was cut into small pieces, snap frozen in liquid nitrogen in cryovials, and stored at −80°C for tissue and plasma extraction. In addition, all implants were recovered, cleaned of tissue debris, dried overnight under vacuum, and stored for future use to measure residual curcumin.

Analysis of residual curcumin in the implants

The rate of curcumin release was determined by measuring residual curcumin in the implants recovered from animals at different time intervals. Implants were dissolved in DCM:ethanol (1:1) and an aliquot of the solution was diluted (1:10) with ethanol containing 20% DCM followed by further dilution in ethanol (1:40). The curcumin concentration was measured using a UV spectrophotometer (Spectrax M2, Molecular Devices) at 430 nm and the rate of curcumin release was calculated by subtracting the residual amount from initial amount per unit time. Initial measurement of these samples by high-performance liquid chromatography (HPLC) gave comparable results and hence all samples were analyzed using UV spectrophotometry (15).

Plasma prolactin

Plasma prolactin was measured by using an EIA Kit (Alpco Immunoassays) following manufacturer’s protocol.

Serum E2 analysis

Serum E2 was analyzed using Roche E170 immunoassay analyzer at the University Hospital’s Clinical Chemistry facility. Estradiol II Reagent Kit purchased from Roche Diagnostics, Inc. was used following the manufacturer’s protocol.

Microsome extraction

Liver (~100 mg) was homogenized in 0.25 M sucrose buffer (pH 7.4) at 3,000 rpm with a polytron homogenizer.
The homogenate was centrifuged at 3,000 × g for 20 minutes at 4°C to separate the nuclear content. The supernatant was further centrifuged at 11,000 × g for 20 minutes at 4°C to remove mitochondrial fraction. The postmitochondrial supernatant was then transferred to ultracentrifuge tubes and centrifuged at 100,000 × g for 1 hour at 4°C to obtain the microsomal pellet that was suspended in 1 mL sucrose buffer, aliquoted and stored at −80°C until further use.

Cytochrome P450 activities
Microsomal proteins were quantified by bicinchoninic acid (BCA) method (17) using BCA Protein Assay Kit (Thermo Scientific). CYP1A and CYP1B1 activities were determined by EROD assay with and without a selective CYP1B1 inhibitor (pyrene). CYP3A4 activity was measured using P450-Glo CYP3A4 assay with Luciferin-IPA following the manufacturer’s protocol by replacing NADPH regenerating system with NADPH (5 mmol/L).

Analysis of plasma and tissue curcumin levels
Plasma (1.5 mL) was pooled from all the animals (n = 6) in each group from 3-week and 3-month time points, and from every third animal from 6-month time point, and 200 µL of 0.5 M sodium acetate was added to reduce the pH to 5. Plasma was then extracted thrice with 3 mL ethyl acetate. Pooled ethyl acetate extract was dried under vacuum. The dried residue was reconstituted in 100 µL of acetonitrile (ACN), one half of which was analyzed by HPLC using Shimadzu liquid chromatography system equipped with LC-10ADVP pump, RF-10AXL fluorescence detector, and a Shimadzu C18 column of 5 µm particles (250 × 4.6 mm). Similarly, liver tissue (~500 mg) from each animal was homogenized in 3 mL PBS (pH 7.4) and 200 µL of 0.5 M sodium acetate was added. The homogenate was then extracted twice with 2 volumes of ethyl acetate. The extract was separated, evaporated under vacuum, and the residue was reconstituted in 1 mL ACN. The ACN solution was filtered through 0.45 µL glass-microfiber filter, evaporated again, and the residue was finally reconstituted in 100 µL ACN, and one-half of it was analyzed by HPLC. The three curcuminoids were separated using ACN and 1% citric acid (adjusted to pH 2.5) mobile phases at a flow rate of 1 mL/min with a gradient elution. The ACN concentration was increased from 0% to 30% in first 5 minutes, followed by an increase to 45% in the next 5 to 20 minutes, and maintained at that ratio until 36 minutes. Curcumin was detected using 410 and 500 nm as excitation and emission maxima, respectively, in the fluorescence detector.

Statistical analysis
Generalized linear model approach was used to analyze the group effect of tumor volume, cube root tumor volume, and tumor number using SAS version 9.2 software. The P value of Shapiro–Wilk normality test for tumor volume and for cube root tumor volume was found to be <0.0001 and 0.7505, respectively. Hence, cube root tumor volume was considered to be distributed normally and was used to calculate statistically significant differences in tumor volumes with different interventions. Tumor-free survival (TFS) was estimated by the Kaplan–Meier method (18). Differences in the survival curves were evaluated through the estimated hazard rates using the unweighted log-rank tests (19). The TFS time was determined as the time from the beginning of the study until the first occurrence of the tumor. All calculations were performed with SAS statistical software (SAS Institute Inc.).

Results and Discussion
We previously showed that curcumin release from polymeric implants follow a biphasic release pattern characterized by an initial burst ranging from 7 to 10 days, followed by a more controlled release phase (13, 15). Implants formulated for this study also reproducibly exhibited biphasic release kinetics with 10.9 mg of curcumin released over a period of 25 days with an average release of 436 ± 5 µg/day from each implant (Fig. 1A). However, the release was much higher (~1.8 mg; 4.5%) on day 1 that decreased slowly with time, as reported earlier (13). The release dropped significantly by 90 days with only 19 mg (~48%) cumulative curcumin released into the systemic circulation in 3 months at a rate of ~127 ± 5 µg/day/implant between 25 and 90 days. Because the release was expected to drop further with time (14), implants were replaced with new implants at ~138 days when average daily drug release dropped to ~94 ± 16 µg/day/implant. Grafting of new implants provided a burst release of curcumin at the time of tumor development and provided a higher daily drug release for the rest of the period to achieve maximum chemopreventive and chemotherapeutic activities of curcumin during the tumor initiation and promotion phases. Furthermore, no significant changes in diet intake or weight gain were observed after the grafting of second implants. The average daily drug release from new implants was 357 ± 38 µg/day/implant from 138 to 180 days, with a total release of 36.1 mg (45%) from all implants combined (Fig. 1A).

It has been previously shown by us and others that the mammary tumors that develop in ACI rats are intraductal carcinoma of the comedo type. In addition, few papillary carcinomas and areas of invasion were present (16, 20, 21). Tumor incidence was measured by weekly palpation for appearance of any tumors starting 12 weeks of E2 treatment. The first tumor in all the E2-treated groups appeared between 95 and 110 days. Although curcumin delivered via diet did not affect the appearance of first tumor (95 days), curcumin implants delayed the tumor appearance by 7 days. However, subsequent appearance of tumors was similar to control treatments, suggesting insufficient doses of curcumin reaching into the systemic circulation. The implants, therefore, were changed at around 135 to 140 days at the peak tumor development stage to provide a burst release of curcumin when ~20% of animals in each group developed tumors. TFS as estimated by the Kaplan–Meier method (Fig. 1B) demonstrated a significant difference (P = 0.03) for all 4 groups, but the comparison of the curcumin diet or the
lactation by the presence of high E2 concentrations in pituitary hormone released during late pregnancy and serum prolactin levels at all the 3 time points. Prolactin is evident from delayed tumor initiation) at least in this tumors and probably had only chemopreventive activity (as evidenced via implants did not have any effect on the growth of tumors). This observation suggests that the curcumin delivered via implants almost similar to the sham implants group (Supplementary Fig. S2). This observation further supported by the tumor multiplicity data (Fig. 3B) as the chemopreventive agents such as indole-3-carbinol are ineffective (Fig. 3B). These results are consistent with studies in the 7,12-DMBA-induce mammary cancer model, in which the chemopreventive agents such as indole-3-carbinol exert maximum effect when given during the preinitiation stage (24, 25) and are more effective in reducing tumor multiplicity than tumor volume (26). Significant reduction in the 7,12-DMBA-induced rat mammary tumor model has been achieved by transdermal delivery of curcumin (27) and oral delivery of curcumin with the aid of cyclodextrin (28). Curcumin implants were highly effective (Fig. 3B) in reducing tumor multiplicity compared with sham implants (2 ± 1 vs. 5 ± 2; dietary curcumin, however, was ineffective (Fig. 3B). These results are consistent with studies in the 7,12-DMBA-induce mammary cancer model, in which the chemopreventive agents such as indole-3-carbinol exert maximum effect when given during the preinitiation stage (24, 25) and are more effective in reducing tumor multiplicity than tumor volume (26). Significant reduction of prolactin release at 6 months (P < 0.001) as compared with sham implants but were ineffective at the 3-month time point (Fig. 2B). Once the old implants were replaced with new implants, the burst release of high curcumin doses again inhibited E2-mediated prolactin release at 6 months (P < 0.05; Fig. 2C). The dietary curcumin, however, was ineffective at all the time points and may need much higher doses to exert chemopreventive activity. These results suggest that release of bolus doses of curcumin at 3 weeks (from initially grafted implants) and at 6 months (from new set of implants grafted after 135 days) were required to significantly reduce plasma prolactin levels.

At the termination of the study, all tumors were measured to determine the average tumor volume (Fig. 3A). Curcumin diet was found to be ineffective as compared with the E2 alone-treated group. However, curcumin implants were found to modestly but significantly (P < 0.05) reduce the tumor volume (by ~35%) as compared with E2 alone treatment. Because curcumin implants as such were not found to affect tumor growth kinetics in this model (Supplementary Fig. S2), it seems that this reduction in tumor volume was because of 3 weeks delay in tumor appearance after the grafting of a new set of implants. This notion is further supported by the tumor multiplicity data (Fig. 3B) as curcumin implants were highly effective (P < 0.0001) in reducing the tumor multiplicity compared with sham implants (2 ± 1 vs. 5 ± 2; dietary curcumin, however, was ineffective (Fig. 3B).
Cure cancer may be in the larder: a novel chemopreventive activity by curcumin implants

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Abstract

Cancer is a chronic disease characterized by uncontrolled growth of cells. Chemoprevention is an attractive approach to inhibit carcinogenesis. This study was designed to investigate the chemopreventive activity of curcumin implants and curcumin diet in breast cancer prevention. Mice bearing a breast cancer (4T1) tumor were treated with curcumin implants or curcumin diet or a combination of E2 implant and curcumin diet. E2 implants alone showed no effect on tumor incidence. Curcumin implants reduced tumor incidence significantly at 3 weeks and 6 months, with no significant difference in tumor multiplicity observed with curcumin implants. There was also a statistically significant reduction in tumor multiplicity in the curcumin diet group. The results from this study suggest that curcumin implants may be a useful tool for cancer prevention, and further studies are required to optimize the delivery system. This study also highlights the potential of curcumin in chemoprevention of breast cancer.

Keywords: cancer prevention, curcumin, breast cancer, chemoprevention, E2 implant.

Background

Chemoprevention is a major strategy to reduce the burden of cancer. Curcumin, a naturally occurring dietary constituent of turmeric, has shown promise in chemoprevention of various cancers. The molecular mechanisms of curcumin are well studied and have been explored in various experimental models. However, the chemopreventive activity of curcumin implants has not been fully explored. The objective of this study was to investigate the chemopreventive activity of curcumin implants and curcumin diet in breast cancer prevention.

Materials and Methods

Mice bearing a breast cancer (4T1) tumor were treated with curcumin implants or curcumin diet or a combination of E2 implant and curcumin diet. E2 implants alone showed no effect on tumor incidence. Curcumin implants reduced tumor incidence significantly at 3 weeks and 6 months, with no significant difference in tumor multiplicity observed with curcumin implants. There was also a statistically significant reduction in tumor multiplicity in the curcumin diet group. The results from this study suggest that curcumin implants may be a useful tool for cancer prevention, and further studies are required to optimize the delivery system. This study also highlights the potential of curcumin in chemoprevention of breast cancer.

Conclusion

Curcumin implants have shown promise in breast cancer prevention. Further studies are required to optimize the delivery system and to confirm the chemopreventive activity of curcumin implants in breast cancer prevention.

Acknowledgments

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References


Figure 2. Plasma prolactin levels of female ACI rats treated with or without a silastic E2 implant along with either 2 sham polymeric implants (2 cm), or 2 curcumin implants (2 cm, 20% load) or curcumin diet (1,000 ppm) for 3 weeks (n = 6; A), 3 months (n = 6; B), and 6 months (n = 6–6; C). Plasma prolactin was measured by using an EIA Kit following manufacturer’s protocol and a P value of <0.05 was considered significant. Groups compared are represented by specific alphabets (*, P < 0.05, **, P < 0.01, and ***; P < 0.001).

in tumor multiplicity observed with curcumin implants, therefore, again suggests that higher doses of curcumin delivered by the next set of implants exerted maximum chemopreventive activity by blunting the initiation of new tumors. It has also been shown that potent inhibition of tumor multiplicity often results from changes in hepatic carcinogen-metabolizing enzymes that are preferentially induced or inhibited by chemopreventive agents to decrease the formation of reactive carcinogen metabolites and enhance carcinogen detoxification (26). This favorable alteration in carcinogen metabolism, then ultimately results in reduced genetic mutations blunting the neoplastic trigger.

Previous studies of estrogen-induced mammary tumorigenesis have shown that E2-mediated mammary tumorigenesis is a 2-step process (27). During the initiation phase, highly reactive carcinogenic E2 metabolites such as estradiol-3,4-quinone bind with DNA, leading to genetic mutations that result in neoplastic transformation of mammary cells (27). Initiation phase is followed by tumor promotion phase, which is mediated by hormonal activity of E2 and 4-hydroxy E2 (4-E2) via their interaction with estrogen receptor α (ERα) and subsequent activation of proliferation signaling (28). Because curcumin implants were found to exert their effect only during the initiation stages, it seems that favorable alteration of hepatic E2-metabolizing cytochrome P450s played a significant role in their efficacy. E2 is metabolized by three distinct P450s to generate different metabolites (shown in Supplementary Fig. S3). E2 metabolism by CYP1B1 results in the formation of 4-E2 metabolite, a potent carcinogen and initiator of carcinogenesis cascades (29). However, E2 metabolism by CYP1A1 (1A1 and 1A2) and CYP3A4 are true detoxification pathways, forming 2-hydroxy E2 (2-E2) metabolite (anti-carcinogenic) and 16α-hydroxy E2 metabolite, respectively (29). Because previous studies showed that modulation of hepatic xenobiotic-metabolizing enzymes affects tumor initiation and tumor incidence (26, 30), we extracted hepatic microsomes and analyzed their CYP1B1, CYP1A1 (1A1 and 1A2), and CYP3A4 activities (Fig. 4A–C).

CYP1B1 activity exhibited time-dependent kinetics with slow sustained E2 delivery by silastic implants (Fig. 4A). Enzyme activity increased significantly after 3 weeks of E2 treatment when compared with untreated 3-week control animals, which decreased to less than the value in untreated controls by 3 months of treatment. At 6 months, an increased CYP1B1 activity was again observed, but the increase was insignificant because of wide variation in the untreated E2-treated animals. Both curcumin diet and curcumin implant group (in the absence of E2 implants) increased 1B1 activity both at 3 weeks and 6 months as compared with untreated control animals (P < 0.05). However, in the presence of E2 implants, dietary curcumin showed no effect on CYP1B1 activity at 3 weeks and 6 months, but increased it significantly at 3 months (P < 0.05). Curcumin implants, however, significantly reduced CYP1B1 activity at all the time points in the presence of E2 implants when compared with sham implants with the E2 group (P < 0.05). Because CYP1B1 is known to be the major xenobiotic-metabolizing enzyme responsible for formation of 4-E2 (31), inhibition of CYP1B1 activity by systemic delivery of curcumin can result in reduced formation of 4-E2, delaying tumor initiation and blunting tumor multiplicity.
CYP1A1 activity also showed time-dependent and exposure-dependent kinetics with E2 treatment. Enzyme activity was found to increase both at 3 weeks (though insignificant) and 6 months (significant) but significantly decreased at 3 months of E2 treatment as compared with untreated animals. Both curcumin diet and curcumin implants increased CYP1A1 activity as compared with untreated animals in the absence of E2 ($P < 0.05$). However, in the presence of E2, dietary curcumin did not have any effect on CYP1A1 activity at 3 weeks but increased the activity significantly at both 3 and 6 months of E2 treatment ($P < 0.001$). Curcumin implants, however, significantly increased CYP1A1 activity at 3 weeks as compared with animals treated with sham implants and E2 ($P < 0.001$).

CYP3A4 activity significantly decreased both at 3 weeks and at 3 months of E2 treatment as compared with control diet group. Although an increasing trend in CYP3A4 activity was observed at 6 months of E2 treatment, because of wide variation in untreated animals, significance was not achieved. Curcumin diet was not found to exert any effect on CYP3A4 activity with or without E2, but curcumin implants significantly ($P < 0.05$) increased CYP3A4 activity at all the time points in the absence of E2. However, in the presence of E2, curcumin implants increased CYP3A4 activity both at 3 weeks and 6 months ($P < 0.05$) when high curcumin concentrations were delivered from new implants (300–400 µg/day/implant) as compared with 3 months of implantation when release was low (~120 µg/day/implant; Fig. 1A).

Because CYP3A4 is a major E2-detoxifying enzyme (31), an increase in its activity leads to increased metabolism and excretion of noncarcinogenic E2 metabolites from the systemic circulation and hence better therapeutic efficacy. Induction of E2 metabolism by curcumin, particularly when delivered via implants, was further supported by circulating E2 levels as reflected by measurement of E2 in serum both after 3 weeks and 3 months, as maximum alteration in enzyme activities was observed at these time points (Fig. 5). As is evident from Fig. 5, administration of E2 by silastic implants significantly increased serum E2 concentration at all the time points as compared with untreated animals. Curcumin diet and implants both did not have any effect on endogenous levels of E2, and serum estradiol concentrations were similar to untreated animals in the absence of silastic E2 implants. However, in the presence of exogenous E2, serum E2 concentration was found to be lower with curcumin implants both at 3 weeks and 3 months when compared with sham implants + E2 group, although the decrease was significant only at 3 months ($P < 0.05$). Dietary curcumin, however, did not decrease the serum levels when compared with E2 alone group but, in fact, significantly increased serum E2 concentration ($P < 0.005$). It is to be noted that an increased serum E2 concentration was observed in sham implants + E2 group when compared with E2 alone group.

Because only curcumin delivered by implants was able to modulate E2 metabolism as compared with dietary route, we further measured curcumin levels both in plasma...
(Fig. 6A) and in liver (Fig. 6B) to determine if these differences in efficacy by both routes were because of differences in curcumin concentrations. Curcumin was undetectable in plasma at all the time points when given by dietary route, consistent with other studies (32), but was found to be 1.1, 0.3, and 0.6 nmol/L in the case of curcumin implants in the absence of E$_2$, after 3 weeks, 3 months, and 6 months, respectively (Fig. 6A). However, significantly higher concentrations of curcumin were observed in the presence of exogenous E$_2$ as compared with E$_2$ only-treated animals.
In the presence of E2 implants, curcumin was detected at levels of 1.4 nmol/L at both 3-week and 3-month time points and 0.9 nmol/L after 6 months of dietary administration and was 2.4, 1.9, and 0.9 nmol/L after 3 weeks, 3 months, and 6 months of E2 treatment with curcumin implants.

Analysis of liver curcumin concentration also revealed a similar trend. Although curcumin was not detected in plasma after dietary administration, it was found in liver tissue at all the time points. In the absence of E2, almost similar levels of curcumin were observed at 3 weeks (9 ± 1 ng/g), 3 months (9 ± 3 ng/g), and 6 months (9 ± 1 ng/g) by dietary curcumin. Curcumin implants, however, resulted in nearly 2-fold higher liver concentration of 16.6 ± 11.7 ng/g after 3 weeks, which decreased slightly to 8.9 ± 3 ng/g after 3 months and increased again to 13.4 ± 8 ng/g at 6 months treatment after replacing the implants at 135 days. As was observed in plasma, significantly increased curcumin concentrations were detected in liver tissue in the presence of E2 implants. Curcumin concentrations of 40 ± 21 ng/g, 32 ± 12 ng/g, and 21 ± 9 ng/g were observed by dietary route after 3 weeks, 3 months, and 6 months of E2 treatment. Somewhat higher curcumin concentrations of 61 ± 28, 35 ± 6, and 32 ± 16 ng/g tissue were observed by implant route after 3 weeks, 3 months, and 6 months of E2 treatment, respectively. Curcumin levels in liver tissue resulting from implant versus dietary routes were not significantly different. However, we have shown in our earlier studies that curcumin levels achieved via burst release from implants (1–12 days) are higher than dietary curcumin (15). Because our studies showed that burst release was a prerequisite to achieve chemopreventive efficacy for inhibition of tumor initiation, as demonstrated by grafting of a new set of implants, these results were in concordance with previously published studies where high doses were established to exploit curcumin’s chemopreventive potential (1, 5). It is to be noted that implants showed efficacy even when the curcumin doses delivered were around 11-, 23-, and 24-fold
lower than the dietary route at 3-week, 3-month, and 6-month time points, respectively. These observations also suggest that not only curcumin (at least via implant route) altered the hepatic metabolism of E2 but the presence of E2 also altered curcumin metabolism to a significant extent. Curcumin is known to get metabolized by both CYP3A4 (33) and UDP glucuronosyltransferase (UGT) (32). It is evident from Fig. 4C that administration of E2 significantly decreased CYP3A4 activity at initial time points, consequently increasing liver curcumin concentration (Fig. 6B). Measurement of UGT activity also showed a similar decrease (data not shown), suggestive of decreased hepatic curcumin metabolism.

Curcumin exerts its chemopreventive efficacy through a wide range of cellular processes, including anti-inflammation, antiproliferation and anti-tumorigenesis (34–36). Most of the activities are attributed to its antioxidant activity and inhibition of cell signaling pathways at various levels, especially through inhibition of NF-κB in preclinical studies (37). Curcumin also induces apoptosis especially in the G2 phase of cell cycle (38, 39) and blocked tumor initiation in chemically induced mammary tumors (40–42). However, it was inefficient in clinical studies up to a dose of 3.6 g per person (43) and more than 8 g was not well tolerated (44). We demonstrate that by providing curcumin directly into the systemic circulation using subcutaneous implants, we were able to increase the tissue bioavailability and chemopreventive efficacy. Even though there are several mechanisms by which curcumin can exert the chemopreventive potential, we had focused this study specifically on estrogen metabolism because these rats develop mammary tumors by an estrogen-mediated mechanism.

Conclusions
Curcumin implants showed diffusion-mediated biphasic release kinetics both under in vitro and in vivo conditions, with a 2-fold higher drug release in vitro as compared with in vivo. Systemic delivery of curcumin via implants significantly reduced the plasma prolactin levels, estrogen-induced mammary tumor burden, and tumor multiplicity as compared with the curcumin administered in the diet.

The enhanced chemopreventive efficacy of curcumin delivered via implants was found to be because of favorable modulation of hepatic CYP450s. CYP1B1 activity was found to be significantly reduced by curcumin implants whereas it was increased by the curcumin diet. CYP1A and CYP3A4 activities were found to be increased significantly by implant route. Together, these data are suggestive of increased metabolism of estradiol to carcinogenic metabolites by curcumin diet and to noncarcinogenic metabolites by curcumin implants. This differential activity was found to be because of different liver and plasma concentrations from both the routes. Curcumin concentration was found to be much higher both in plasma and in the liver when delivered via implants as compared with the curcumin diet.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
Inhibition of Mammary Carcinogenesis by Curcumin Implants


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