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Development and evaluation of polymeric implants of curcumin for enhanced chemopreventive activity.

Shyam Sunder Bansal
University of Louisville

DEVELOPMENT AND EVALUATION OF POLYMERIC IMPLANTS OF CURCUMIN FOR ENHANCED CHEMOPREVENTIVE ACTIVITY

By

Shyam Sunder Bansal
M.S., NIPER, 2007
M.S., University of Louisville, 2009

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A Dissertation Approved on

July 14, 2011

by the following Dissertation Committee:

Ramesh C. Gupta, Ph.D.

Jason Chesney, M.D., Ph.D.

Teresa Fan, Ph.D.

Haribabu Bodduluri, Ph.D.

Roland Valdes, Ph.D.

DEDICATION

This dissertation is dedicated to my parents and my wife for their

constant support and encouragement

to achieve my goals

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First of all, I wish to thank great almighty God for blessing me with enough patience, knowledge determination, confidence and strength to accomplish my goals and wish the same mercy for my future endeavors.

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ABSTRACT

AN IMPLANTABLE DRUG DELIVERY CONCEPT IN CANCER CHEMOPREVENTION

Shyam Sunder Bansal

August 08, 2011

Curcumin, a plant derived compound has shown significant potency against various chronic diseases like cancer in cell culture and animal studies. However, the introduction of this compound into clinical setting is limited by its poor oral bioavailability and thereby requires high doses for efficacy. We hypothesized that localized/systemic delivery of curcumin by polymeric implants can improve its efficacy by bypassing the oral route and by restricting majority of the drug at the tumor site. Therefore, we developed a polymeric implantable drug delivery system using poly (ε-caprolactone) as the polymer and optimized it with respect to polymer molecular weight, drug load and formulation additives both under *in vitro* as well as under *in vivo* conditions so as to obtain a desired drug release profile.

Drug release was found to be proportional to surface area and drug load for up to 10% concentration. The presence of water-soluble polymers like polyethylene glycol (PEG) at 35% w/w composition was also found to increase the drug release with *in vivo* release being 1.8-2 fold higher than *in vitro* release. Higher plasma and brain curcumin concentrations with almost similar levels in liver were observed by these implants but at

25-30 fold lower doses than dietary curcumin even after 3 months. Furthermore, these implants were found to provide a controlled/predictable release ranging from months to years.

Analysis of biochemical parameters of liver and kidney function revealed non-toxicity of the implant formulation as well as of curcumin administered continuously into systemic circulation by these implants. Although a mild to moderate inflammatory reaction was observed at the local site of implantation, yet no toxic effect on health or physical well being of rats was observed. Moreover, systemically administered high doses of curcumin (by implants) were found to be more efficacious in modulation of cytochrome P450 enzymes like CYP1A1, CYP3A4 and CYP1B1 as well as in inhibiting E_2-mediated mammary tumorigenesis in ACI rats. These implants were found to reduce the tumor burden significantly by 35% and tumor multiplicity by 70% *via* favorable alteration of E_2 metabolism suggestive of curcumin's potent chemopreventive activity when delivered *via* implants as compared to diet.

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CHAPTER I

INTRODUCTION

Breast cancer is the second leading cause of cancer deaths in US and accounts for 15% of all cancer deaths. It is estimated that 1 in 8 women in the United States will develop breast cancer in their life time and 1 in 30 will die from it. Statistics further showed that breast cancer death rates remained almost constant from 1930 to 1990 and decreased by 29% in 1990 to 2006 (American Cancer Society) (1). This decrease in breast cancer incidence was due to the advancements in breast cancer screening and treatment strategies. Currently, surgery, radiation and systemic therapy with chemotherapeutic agents are the most widely used approaches for breast cancer treatment. Surgery and radiation are undesirable by women due to obvious reasons and it is only the systemic therapy that provides few alternative treatment regimens for breast cancer. Systemic therapy involves use of biologics like trastuzumab (her2/neu monoclonal antibodies), chemotherapy (with paclitaxel, docetaxel), hormone therapy (with tamoxifen) for estrogen receptor-positive breast tumors or metronomic chemotherapy involving low, less toxic doses of anti-angiogenic agents (2).

However, the use of these regimens is limited either due to their low bioavailability, high toxicity, significant first-pass metabolism, high doses and/or poor patient compliance. Due to these limitations other safe drugs or therapeutic regimens are often desired (2).

Since a typical carcinogenesis process involves many environmental, dietary, occupational, and epigenetic factors that determine its long latent period; intervention with phytochemicals that have little or no toxicity can provide an alternative strategy for controlling the initiation and progression of this disease. As a result, studies initiated by the National Cancer Institute have led to the screening and identification of thousands of natural compounds, of which a few dozen (like curcumin, resveratrol, epigallocatechin gallate (EGCG), ellagic acid, quercetin, and indole-3-carbinol) exhibited potent chemopreventive/anti-tumor activity in cell-culture as well as in some animal studies. These agents were found to block the cancer progression by affecting all the stages of carcinogenesis, including transformation, proliferation and invasion of tumor cells. Their long-term use in folklore medicines from historic times further demonstrate their safety profile and their potential for long-term use in normal as well as high-risk populations (3). These chemopreventives are widely distributed in almost all the colored fruits and vegetables and ≥5 servings of fruits and vegetables per day are recommended by American Cancer Society for their chemoprevention efficacy against various diseases, including cancer (1). However, less than 1 in 4 American adults eat the recommended servings and there has been very little improvement in the consumption of fruits and vegetables since 1990s (American Cancer Society) (1). Therefore, other alternative means are required for the administration of active chemopreventive compounds and their usage in day to day life.

2.0. Curcumin: A Potent Chemopreventive

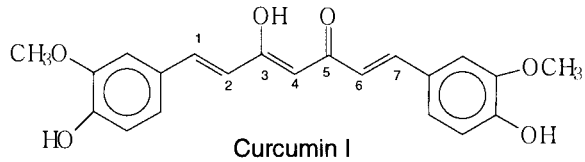
Although, a number of potent chemopreventives have been identified from plant sources, curcumin, a principal bioactive component of *Curcuma longa* (turmeric), represents one of the most investigated phytochemicals. Pubmed shows ~4000 hits on using curcumin as the search string with ~1200 hits from last 2 years alone. There are 3

major curcuminoids that constitute curcumin: curcumin (curcumin I, 75%), demethoxycurcumin (curcumin II, 20%) and bisdemethoxycurcumin (curcumin III, 5%) (Figure 1.1). Research over last two decades has shown curcumin to be a potent anti-oxidant, anti-inflammatory, anti-proliferative, antimetastatic, anti-angiogenic, anti-diabetic, hepatoprotective, anti-atherosclerotic, anti-thrombotic, and anti-arthritic agent (4, 5). Various cell culture studies have shown that it inhibits various protein kinases (like EGFR, IKK, JAK2, PKC, PKA), transcription factors (like NF-kB, AP-1, STAT, PPARγ) and enzymes (like GST, hemoxygenase, xanthine oxidase) and various others (Figure 1.2) (4-7) responsible for its variety of potent activities. However, 3 main mechanisms have been postulated to be responsible for curcumin's anticancer/chemopreventive activities.

(i) Modulation of hepatic cytochromes (8).

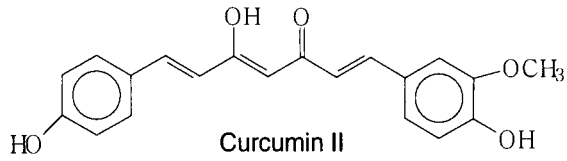
(ii) Induction of antioxidant response pathways (9).

(iii) Inhibition of NF-kB activation (6, 10).

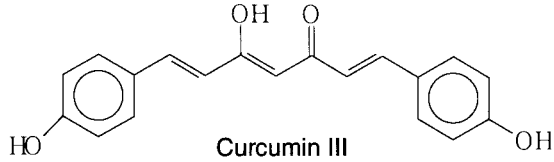


Curcumin I

1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,3,6-heptatriene-5-one-3-ol;



Curcumin II



Curcumin III

Figure 1.1. Structures of curcuminoids.

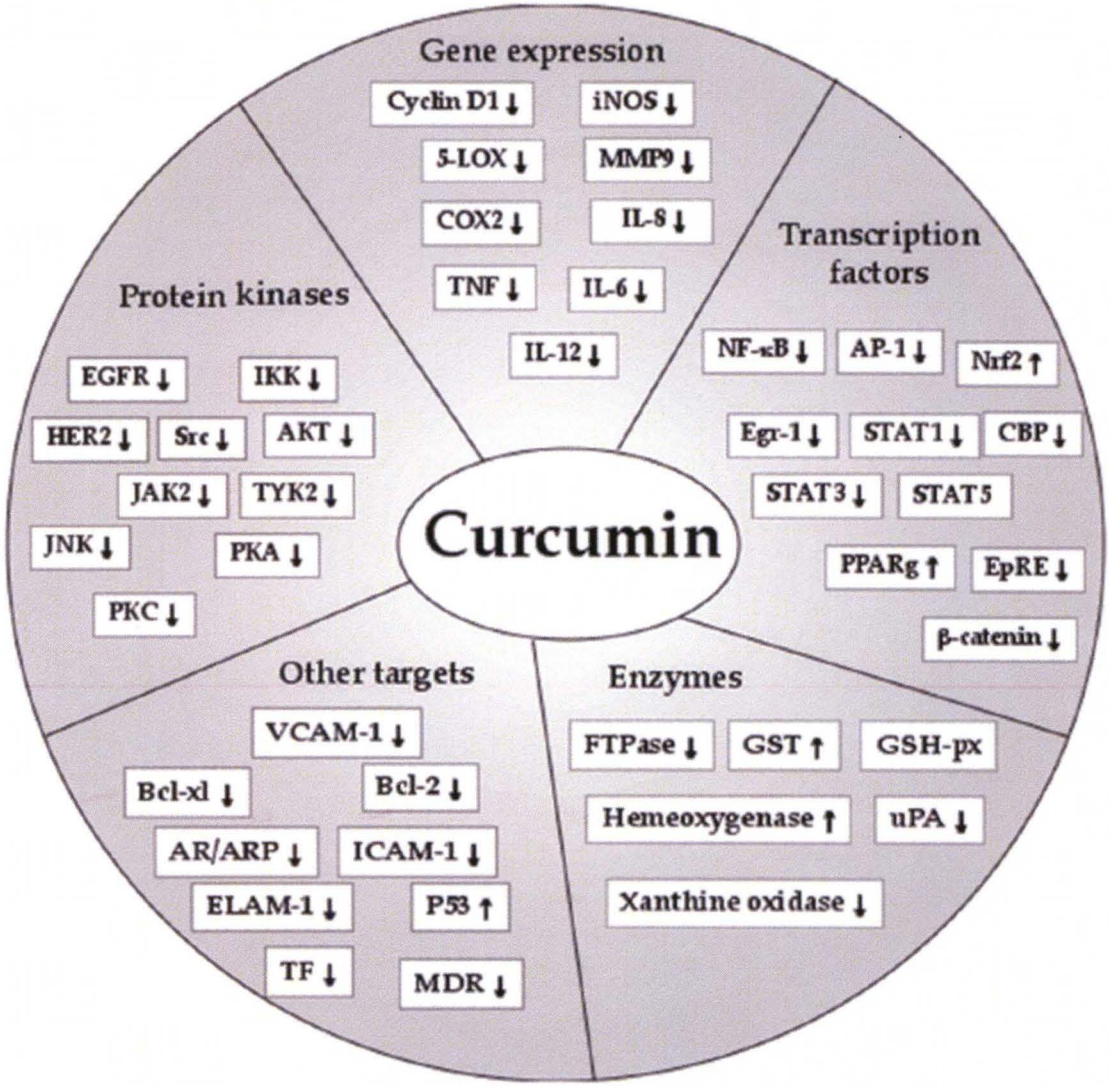


Figure 1.2. Various intracellular signaling molecules affected by curcumin (5).

2.1.1. Modulation of Hepatic Cytochrome P450s

Curcumin is well known for its potential to inhibit carcinogenesis induced by chemical carcinogens, at both initiation and progression stages by altering various xenobiotic metabolizing enzymes in the liver (11). It has been found to interact with pregnane X receptor (PXR) to induce expression of CYP3A4 by which not only it alters the metabolism of various other xenobiotics but also induces its own metabolism in

conjunction with UGT (12). It is also both an inducer as well as a competitive inhibitor of CYP1A1 inhibiting bioactivation of environmental carcinogens like benzo[a]pyrene (B[a]P) (13). It is a potent ligand to aromatic hydrocarbon receptor (AhR) and interacts with it in the cytoplasm (Figure 1.3). AhR exists in cytosol in complex with heat shock protein 90 (hsp90) and gets activated on interacting with curcumin like ligands. Liganded AhR then translocates into the nucleus and interacts with AhR nuclear translocater (ARNT). The complex then interacts with the xenobiotic response elements (XRE) or dioxin response elements (DREs) to initiate the expression of CYP P4501A1 genes (14).

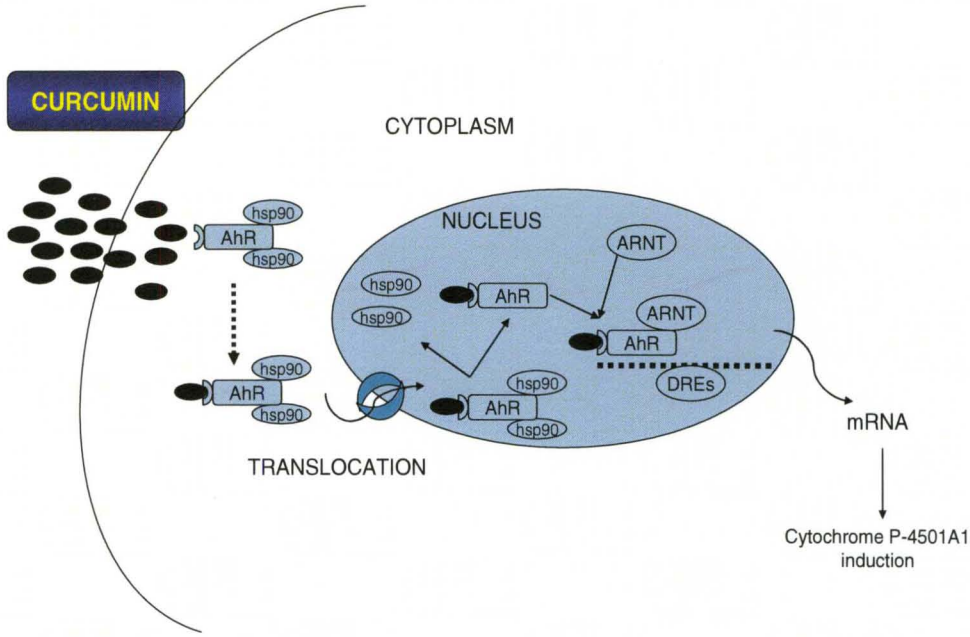


Figure 1.3. Schematic overview of CYP 1A1 induction by curcumin via AhR pathway

2.1.2. Activation of Nrf2 Pathway

Curcumin is also a potent inducer of endogenous antioxidants via activation of Nrf2 pathway to strengthen body's defenses against reactive oxygen species (ROS) (11) (Figure 1.4). Expression of endogenous antioxidants, like GSH, is controlled by NF-E2-related factors 1 and 2 (Nrf1 and Nrf2) that bind to antioxidant response elements (AREs) and activate various genes. The activity of Nrf2 is controlled by inhibitory effects

of a cytoskeletal protein keap1 and the complex exists in an inactive form in the cytosol. In conditions of environmental stress, cigarette smoke, reactive oxygen species or metals, Nrf2 gets phosphorylated which then translocates into the nucleus. In the nucleus, Nrf2 in association with bZIP family of transcription factors interacts with AREs to induce expression of endogenous antioxidants, xenobiotic-detoxification enzymes, transporters and proteasomes (9).

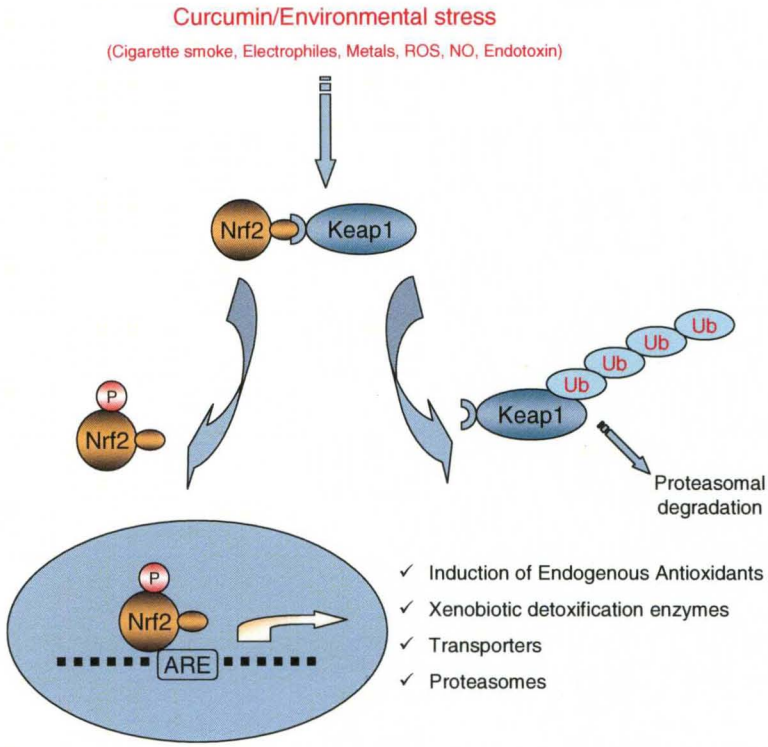


Figure 1.4. Schematic overview of Nrf2 pathway activation by curcumin

2.1.3. Blockade of NF-kB Pathway

Curcumin's potent anti-inflammatory activity has been shown to be due to the inhibition of IκB kinase required for the activation of NF-kB, an important transcriptional regulator of inflammatory pathways involved in carcinogenesis and various other pathological conditions (6, 10) (Figure 1.5). NF-kB belongs to a family of 'rapid acting' primary transactivation factors that regulate various inflammatory and immune responses in the

body. NF-kB exists as an inactive heterodimer or homodimer of p50, p52 or p65 sequestered with Inhibitory kB protein (IkBs). Phosphorylation and subsequent degradation of IkB by IkB kinase result in the activation of NF-kB which then translocates into the nucleus to initiate transcription of various genes regulated by it. Curcumin has been found to be a potent inhibitor of IkB kinase and hence inhibits the activation of NF-kB in response to various inflammatory stimuli. Since NF-kB plays an important role in the initiation and progression of carcinogenesis, its inhibition by compounds like curcumin has been postulated to be one of the important mechanisms of its anticancer activity (15).

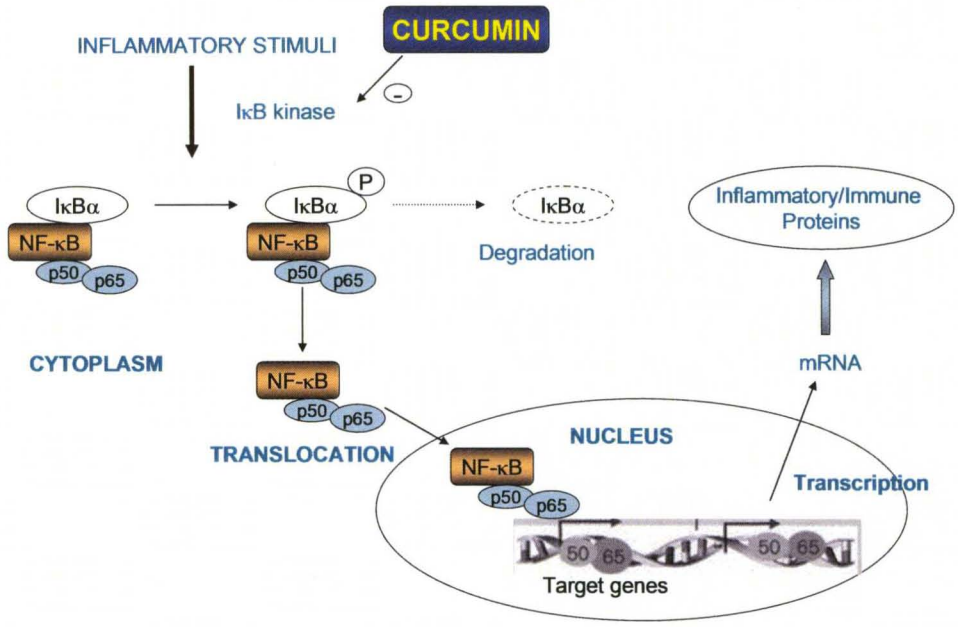


Fig. 1.5. Schematic overview of curcumin mediated NF-κB inactivation via inhibition of IκB kinase

2.2. Physico-chemical Properties of Curcumin

Curcumin is a yellow-orange crystalline powder first isolated in 1815 from rhizomes of *Curcuma longa* (turmeric). Its structure was deduced in 1910 by Lampe et al. and was confirmed by synthesis in 1913 by lampe and Milobedzska (16). It melts at 180-183 °C and is practically insoluble in water but soluble in ethanol, dimethylacetamide,

dimethylsulfoxide and acetone (17). It exhibits a brilliant yellow hue at pH 2.5-7 and red at alkaline pH (17). Depending upon the solvent used, it absorbs at 420-430 nm in visible region and exhibits three acidity constants *viz* pK_{a1} = 8.38±0.04, pK_{a2} = 9.88±0.02 and pK_{a3} = 10.51±0.01 (18). Chemically, it is bis-α,β-unsaturated β-diketone and exists in equilibrium with its enol tautomeric form (Figure 1.). In acidic and neutral aqueous solutions bis keto form, a H-atom donor, is predominant while basic solutions shift the equilibrium towards enolate anion which acts as a strong e^- donor at basic pH (17). In acidic/neutral conditions, the e^- density of two adjacent –C=O oxygens get localized and the C-H bonds in central $–CH_2$ group in hepta-di-ene chain becomes very weak. These labile hydrogens can then react with other radicals to exert an antioxidant activity. However, at pH>8, due to enolate form, the reaction with the free radical blunts and the phenolic part acts as an e^- donor that can scavenge alkyl phenoxy radicals. Hence, this keto-enol tautomerism confers curcumin with its free radical-scavenging activity which is further substantiated by intermolecular H bonding predominant in its enolate form (19). Furthermore, it is reported that potent antioxidant activity of curcumin is conferred by its two terminal methoxy phenol groups (19). However, empirical linear energy relations showed oxidation potential of curcumin to be 0.77V which is considerably higher than other potent phenol group containing antioxidants like vitamin E (~0.48V), vitamin C (~0.28V), methyl gallate (~0.52V) or quercetin (~0.33V) (19). According to Jovanovic et al., curcumin's potent antioxidant properties might not only be due to its methoxy phenol groups and other structural features, like keto-enol tautomerism, also contribute significantly (19). Furthermore, hydroxyl groups on the aromatic rings and the diketone functionality both are required structural features for the induction of phase II detoxifying enzymes (20). However, curcumin is also highly unstable at basic pH and ~90% degrades within 30 mins to form trans-6-(4'-hydroxy-3'-methoxy phenyl)-2,4-dioxo-5-hexanal, vanillin, ferulic acid and feruloyl methane (21). But its stability increases on

supplementing the basic solutions with albumin or with blood/plasma and in presence of antioxidants such as ascorbic acid, N-acetylcysteine or glutathione (22).

2.3. Curcumin Pharmacokinetics

Although the structural attributes of curcumin make it a potent anti-oxidant, they also render it poorly water soluble. As a consequence, it exhibits solubility-limited bioavailability, which makes it a class II drug in the Biopharmaceutical Classification System (BCS) (23). Furthermore, due to its rapid intestinal and hepatic metabolism, approximately 60-70% of an oral dose of curcumin gets eliminated in the feces (22). In both pre-clinical as well as clinical studies, even high doses of orally-administered curcumin (8-12 g daily) resulted in very low concentrations in the plasma levels (<1 μg/ml) that were not high enough to exert any significant pharmacological or therapeutic activity (table 1.1) (24). In rats, curcumin administered as an aqueous suspension (2g/kg) provided a maximum plasma concentration of 1 μg/ml within 1 h, and dropped rapidly to undetectable levels within 5 h (25). When curcumin was administered by parenteral routes like i.v., ~50% was found to be eliminated in bile within 5 h. It has been found that curcumin undergoes rapid metabolism in the liver to form various active and inactive metabolic products that are further converted into excretable glucuronide and sulfate conjugates. Curcumin provides easily accessible phenolic –OH and -OCH_3 sites (Figure 1.1) to form conjugates with glucuronides and sulfates that can be deconjugated by glucuronidases and sulfatases, respectively (12). It has been suggested that this biotransformation of curcumin occurs during absorption or by first-pass metabolism which then undergoes enterohepatic recirculation (26). These results were further supported by Shoba et al (25), who showed that co-administration of piperine (20 mg/kg), a potent inhibitor of glucuronidation in the liver and gastro-intestinal tract, significantly increased the curcumin bioavailability by 20 fold in humans.

Table 1.1

Examples of some pre-clinical (A) and clinical (B) studies reported with curcumin.

A: Preclinical Studies

Animal	Route	Dose	Findings	Refs.
Rats	oral	1g/kg	• Poorly absorbed • 75% excreted in feces	(27)
Rats	oral	2% diet	• 12 nM in plasma	(28)
Mice	i/p	100 mg/kg	• 2.25 µg/ml in 15 mins • Disappeared within 3h	(22)
Rats	i/v	40 mg/kg	• Disappeared within 1 h	(29)

B: Clinical Studies

Route	Dose	Findings	Refs.
Oral (n=34*)	1-4 g/day for 6 months	• No reduction in peripheral biomarkers of inflammation • No improvement in cognitive performance in alzheimer's patients.	(30)
Oral (n=21*)	8 g/day until disease progression	• 1 patient showed stable disease (<18 months) and one showed tumor regression with an increase in serum cytokines 22-41 ng/ml peak plasma levels	(31)
Oral (n=25*)	8 g/day for 3 months	• ~1.77 µM plasma conc. Peaked at 1-2 h and declined within 12 h	(32)
Oral (n=12*)	450-3,600 mg/day for 1 wk prior to surgery	• Poorly available, insufficient hepatic levels for inhibition of hepatic metastasis from colorectal cancer	(33)

* denotes number of patients in the trial

These limitations of low solubility, rapid metabolism and hence low bioavailability have limited the therapeutic success of curcumin largely to cell culture systems and elicited only limited efficacy in various preclinical and clinical studies. In order to overcome this problem, several advanced drug-delivery systems have been designed to provide localized or targeted delivery of curcumin. Various drug-delivery systems – such as nanoparticles, liposomes, microparticles and microemulsion – have been demonstrated

to significantly enhance the preventive/therapeutic efficacy of curcumin by increasing their bioavailability and targetability which are discussed below (34).

Curcumin Drug Delivery Systems

2.4. Curcumin Nanoparticles

The advent of nanotechnology has enabled the development of various nano-particulate drug delivery systems that can enable the formulation of hydrophobic drugs like curcumin (table 1.2) which earlier was a conundrum for the formulation scientists (35). According to the National Nanotechnology Initiative (NNI), nanoparticulate (NP)-delivery systems contain encapsulated, dispersed, adsorbed, or conjugated drugs within a particle size range of 1-100 nm (36). These delivery systems have gained immense popularity in the last decade due to their potential to improve the therapeutic index of the encapsulated drugs either by protecting them from enzymatic degradation (37), by altering their pharmacokinetics (38), by blunting their toxicity (39) or by providing controlled release over extended periods of time (40).

Upon oral administration, colloidal particles like nano/micro-particles, are absorbed in their intact form *via* lympho-epithelial M cells of peyer's patches. These particles bind to the apical side (towards the lumen) of M cells gets internalized, and are subsequently shuttled to lymphocytes (41). These colloidal carriers transit slowly in the gut which increases the local concentration gradient across the absorptive segments of the intestine, further enhancing the absorption rate (42). NPs as drug delivery vehicles also enables passive targeting in tumors and other inflamed tissues due to increased vascular leakiness that results because of increased production of cytokines and angiogenesis cascades at these sites. In majority of solid tumors, the vascular cut-off

pore size ranges between 380-780 nm (43) whereas normal vasculature is impermeable to particles larger than 2-4 nm (44, 45). Such a vast difference in vascular permeability enables the passive targeting of tumor and inflamed tissues by NPs and leads to their accumulation resulting in an enhanced permeation and retention (EPR) effect (43). As a consequence of this passive accumulation at target sites, the concentration of the drug at healthy tissues is correspondingly lower, thereby blunting the intensity of side effects. However, it is noted that for patients with other patho-physiological conditions that are associated with leaky vasculatures, drug delivery by NPs could result in distribution to multiple sites, thereby blunting (to some extent) selectivity for tumor tissues. Such delivery systems are particularly effective in testing and developing new chemical entities including natural compounds like curcumin that possess sub-optimal physicochemical and pharmacokinetic properties to be developed as new drug candidates.

Being lipophilic curcumin partitions/encapsulates into the hydrophobic core of amphiphilic polymers or phospholipids of NPs which not only enhance its bioavailability but also increase its stability by protecting them from the influence of outside environment (40). One of the most investigated method for preparing such NP formulations is emulsion-diffusion-evaporation method which involves solubilizing the drug and/or polymer (Poly(lactic-co-glycolic acid)) in any organic solvent like ethyl acetate, followed by its drop wise addition into an aqueous phase that contains a suitable stabilizer to result into an emulsion. The emulsion can then be homogenized and diluted with a large quantity of water so that solvent diffusion can result in nano-precipitation. This method provides uniformly sized (120-240 nm) spherical NPs of curcumin (Figure 1.6), and since solubility of the incorporated drug plays a pivotal role in determining encapsulation efficiency, stabilizers with lower drug solubility were found to

be better candidates for achieving high drug encapsulation (46). *In vivo* studies in rats showed that curcumin NPs increased curcumin bioavailability by 26-fold as compared to oral curcumin suspension and by 9-fold as compared to a curcumin suspension administered in conjunction with piperine (46). Furthermore, similar PLGA NPs, prepared by Anand et al. (47) using F-68 as the solubilizer, were found to possess similar efficacy as free curcumin in killing tumor cells but a higher potency in inhibiting NF-kB activation in cell culture, compared to free curcumin. The authors of this study also claimed superior bioavailability from curcumin NPs; this claim, however, is difficult to assess as curcumin was administered to the mice *via i.v.* route where bioavailability does not come into play. Nonetheless, an increased half-life of the curcumin in plasma was evident (47).

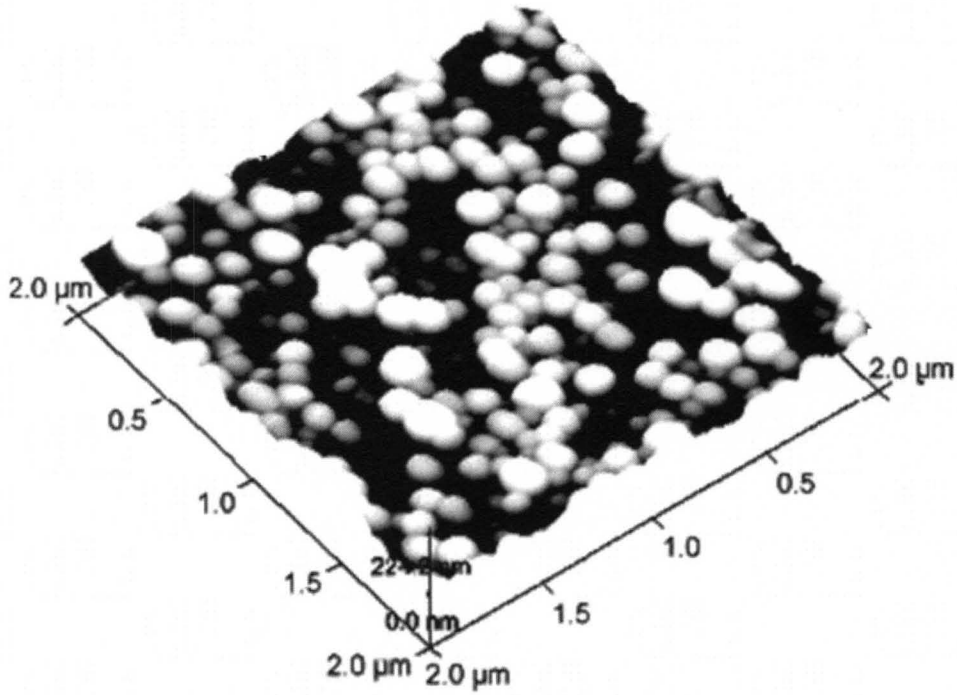


Figure 1.6. Atomic force microscopy (AFM) image of curcumin-loaded PLGA nanoparticles prepared by emulsion-diffusion-evaporation method (From shaikh et al., reproduced with permission) (46).

Curcumin NPs can also be prepared using other copolymers like N-isopropylacrylamide (NIPAAM), N-vinyl-2-pyrrolidone (VP) and poly(ethyleneglycol) monoacrylate (NIPAAM (VP/PEG A)) (48). These NPs possess very low polydispersity with an average particle size of 50 nm that enables them to freely permeate into different pancreatic cancer cell lines. Although, these curcumin NPs were found to be equally efficacious as free curcumin in cell culture but had an added advantage of their direct injectability into the systemic circulation, thereby bypassing the oral route (48).

Another method to prepare curcumin NPs is by anionic polymerization-solvent-evaporation method (49). This method involves drop-wise addition of a butylcyanoacrylate monomer solution into a constantly stirred acidic ethanol solution containing a suitable surfactant and sodium sulfate. At the critical micelle concentration (CMC), surfactant molecules aggregate together to form a swollen micellar structure containing multiple monomer units. Polymerization of monomer units occurs inside these micelles, forming primary polymer particles that grow in size to form NPs. Curcumin or any other chemopreventive can be added during or after the addition of monomer solution to achieve efficient encapsulation during the growth phase. This method provides uniform NPs (PDI of 0.23-0.27) of 160-240 nm, with particle size directly related to monomer concentration and inversely related to surfactant concentration (49). Furthermore, it also results in the formation of a highly porous structure with a very high surface area that can be loaded with hydrophobic drugs like curcumin (50). These NPs were found to provide higher drug release under *in vitro* conditions at acidic pH compared to physiological pH, demonstrating their ability to efficiently deliver their cargo inside the cells after degradation by lysosomes, where conditions are more acidic (49).

The other advantage of using polymeric NPs is their amenability to alterations of surface properties. Different functional groups like thiols can be covalently or non-covalently conjugated with the polymeric chains to increase or decrease the mean residence time of the nanoparticles in the gastrointestinal mucosa. Grabovac *et al.* prepared such PLGA NPs modified at the surface with thiolated chitosan (40). Thiolated chitosans owing to their –SH groups interact with mucus to form disulphide linkages conferring them with highly muco-adhesive properties and hence an increased residence time (51). Furthermore, due to various inter- and intra-molecular disulfide bonds between chitosan molecules, a tight 3D structure results providing a controlled release (51). Other mechanisms like reversible opening of tight junctions and inhibition of efflux P-gp pumps have also been demonstrated to be associated with these thiolated chitosans (52). Although thiolation increases the mean residence time of the coated NPs on the mucosa, it also increases the particle size with decreased encapsulation efficiency of drugs as compared to unmodified NPs (40). The size of curcumin NPs was found to increase from 284-420 nm to 817-960 nm on chitosan coating with half the entrapment efficiency limiting the drug-loading capacity of the thiolated NPs (40).

Another variant of modified NPs is formulation of multi layered polyionic/polymeric shells encapsulating NPs containing drugs. These polyelectrolyte shells are formed as layers over the surfaces of NPs to alter their cell uptake, to attach tumor targeting agents, to increase stability, and/or to control their loading/release characteristics (53). These layered NPs have been demonstrated using gelatin as the polymer and can be prepared by a two-step desolvation method followed by formation of layered poly-ionic shells (54). First, the gelatin NPs are prepared by precipitating gelatin from an acidified solution by slowly adding acetone and then cross linking gelatin with glutaraldehyde. Then, an aqueous solution of these NPs can be coated with polyionic shells by the sequential

addition of polyanions (polystyrene sulfonate, poly-L-glutamic acid or dextran sulphate) and polycations (polyallylamine HCl, poly-L-lysine, or protamine sulphate) at pH 6. Since gelatin is positively charged at acidic pH, a polyanionic layer forms first followed by a polycation layer. Once prepared, these NPs can be further added to curcumin solution to adsorb curcumin at their surface via hydrophobic interactions that develop between curcumin's phenol groups and gelatin's amino acids like proline (53).

Such multi-layered NPs can also be modified for the targeted delivery of chemopreventives. In such nanostructures, polymeric layers with the entrapped chemopreventives encapsulate a magnetic iron core that acts as a targeting system (55). Efficacy of such multi-layered NPs of curcumin was demonstrated by Koppolu et al, (55) using poly (NIPAAM) and PLGA as polymers. In this approach, NIPAAM undergoes free radical polymerization onto the magnetic core *via* covalent coupling with a silane reagent. The resultant NPs can then be coated with PLGA using a double emulsion solvent evaporation method, yielding NPs of 500-1000 nm size that can be used to deliver both hydrophilic and hydrophobic chemopreventive compounds simultaneously. The hydrophilic compounds can be loaded into the poly (NIPAAM) layer, and the hydrophobic drugs can be loaded into outer PLGA layer (55). However, attachment of multiple poly (NIPAAM) particles at the surface of PLGA particles as well as encapsulation of multiple poly (NIPAAM) particles in the PLGA layer (as opposed to the encapsulation of a single particle) raises some concerns regarding control and the success of the formulation method.

Targeted delivery of chemopreventives can also be achieved by conjugation of NPs or drugs with ligands like folic acid that can recognize some specific surface attributes of target cell types. Different cancer types often over-express some specific epitopes or

receptors (56) and bio-conjugation of chemopreventives to ligands having high specificity for these unique surface receptors can help in achieving their targeted delivery to any cancer type. Salmaso et al. (57) demonstrated targeted delivery of curcumin by attaching folic acid (as a ligand) to the polymeric carrier. Presence of folic acid enabled these NPs to undergo clathrin independent endocytosis into cells that specifically over express folic acid receptors. This formulation involved conjugation of PEG, covalently linked to folic acid on one end with isocyanate group of hexamethylene which is further linked to a cyclodextrin curcumin complex on the other end. Hexamethylene is used as a linker to decrease the steric hindrance of bulky PEG chains with cyclodextrin curcumin complex where cyclodextrin was used to bind curcumin into its cavity and to enhance its solubility. These conjugated complexes of curcumin were found to be i) 3,200 times more soluble, ii) ~12 times more stable, iii) 2 times more specific, and iv) 45 times less degradable at pH 7.2 (the degradation rate constant decreased from $321X10^{-4}$ min^{-1} to $7X10^{-4}$ min^{-1}) compared to curcumin alone (57). However, an insufficient cell uptake led to limited beneficial effects of this bio-conjugate and further biological investigations are required to demonstrate efficient drug release from the conjugates into the tumor cells.

2.4.1 Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) are prepared by using lipids instead of polymers and have also shown significant potential for the delivery of lipophilic compounds like curcumin (58). SLNs were first introduced in mid 1990s as novel drug delivery systems (59) capable of protecting the labile drugs from light/pH/heat mediated degradation, controlled release and excellent biocompatibility/toleratability (60). These are spherical lipid NPs with a high specific surface area that can be easily modified to i) attain a favorable zeta potential, ii) pseudo zero-order kinetics, iii) rapid internalization by cancer

cells and iv) impart stealth properties to lessen uptake by the reticulo-endothelial system (RES). These properties make them highly versatile drug delivery systems for a variety of compounds with different physicochemical and pharmacological properties (58). Their lipophilic character enables them to cross the blood brain barrier (BBB), providing a viable alternative vehicle for the delivery of less lipophilic drugs that cannot cross the BBB (35). Furthermore, biological origin of lipid component of these SLNs renders them less toxic as compared to polymeric NPs (61). This drug delivery carrier not only protects the entrapped drug from photochemical or pH mediated degradation but also enables drug targeting and easy large scale production (62, 63). Such characteristics make SLNs as suitable drug delivery carriers for curcumin (table 1.2) and other chemopreventives like resveratrol, and β-carotene which owing to their lipid solubility gets localized in the bilayer membrane of lipid vesicles/NPs and results in enhanced bioavailability. Initially, hot homogenization and warm microemulsion techniques were used for the preparation of SLNs but later other advanced techniques like high pressure homogenization, solvent emulsification evaporation/diffusion, high speed stirring, double emulsion method and ultrasonication were introduced (35).

Curcumin SLNs can be formulated using dimyristoyl phophatidylcholine (DMPC) via extrusion through a 0.2 μm filter (64). These vesicles were surface modified by L-glutamic acid, N-(3-carboxy-1-oxopropyl)-1, 5-dihexadecyl ester, and PEG to increase their uptake by macrophages. Since, macrophages produce ROS that leads to oxidative damage and inflammatory responses, curcumin delivery to these macrophages can result in its maximal anti-inflammatory action. Sou et al. (64), has reported localization of curcumin SLNs in macrophage rich sites such as bone marrow, spleen, and liver even at 6 h after the injection, demonstrating their preferential uptake by macrophages and their considerable ROS scavenging potential equivalent to 160 to 1,050 SOD units when

analyzed by a hypoxanthine and xanthine oxidase system (64). Although an initial decrease in white blood cells, red blood cells and platelets was observed with these vesicular NPs, the levels of these blood components recovered within 3 h demonstrated absence of any acute toxic response of body towards these delivery vehicles. The potential of this system to deliver curcumin to different tissues was further demonstrated by the presence of yellow fluorescence of curcumin in tissue samples of animals, as detected by confocal microscopy (64). One concern with this approach, however, involves an increase in curcumin release from these vesicles at room temperature (20-30°C), suggesting a possible problem with the retention of entrapped curcumin during long storage.

Table 1.2.

Polymeric and solid lipid nanoparticle formulations of curcumin.

Formulation	Method	Particle Size (nm)	Encapsulation Efficiency (%)	Advantages	Disadvantages	Refs.
PLGA NPs of curcumin for oral administration.	Solvent Evaporation Diffusion	120-240 (PDI = 0.31)	77	• 26 fold increased oral bioavailability as compared to oral curcumin suspension.		(46)
PLGA and PEG NPs of curcumin for parenteral administration.	Nano-precipitation	80-90	97.5	• Increased biological half-life of curcumin. • Enhanced inhibition of TNF induced NFkB activation as compared to free curcumin.		(47)
NIPAAM NPs of curcumin containing PEG monoacrylate.	Micellar Aggregation	~50	>90	• Direct systemic administration. • Increased efficacy at lower doses.		(48)
PLGA NPs of curcumin coated with thiolated chitosan.	Emulsion Solvent Evaporation	578±67 (pH 7.4)	28	• ~3.3 fold increased residence time on gastric mucosa.	• Thiolation leads to increased particle size.	(40)
Butylcyanoacrylate NPs of curcumin coated with poloxamer 188.	Anionic Polymerization Solvent Evaporation	160-240 (PDI ~0.25)	78	• Highly porous structure. • Higher drug release at acidic pH for intra-cellular delivery to lysosomes.		(49)
NIPAAM NPs of curcumin multi layered with PLGA.	Free Radical Polymerization Double Emulsion Solvent Evaporation	500-1000	49.5	• Potential to deliver both hydrophilic as well as hydrophobic drugs simultaneously.	• Probability of encapsula-–tion of multiple particles inside PLGA layers	(55)
Surface modified DMPC SLNs for parenteral administration.	Extrusion through 0.2 μM filter	187±53	97	• Increased uptake by macrophages for maximal anti-inflammatory activity.	• Cannot be stored for longer periods of time.	(64)

2.5. Liposomes

Liposomes are the spherical bilayer vesicles with an aqueous interior formed by the self association behavior of amphiphilic phospholipids with cholesterol molecules. This self associating behavior of phospholipids originates from their tendency to shield their hydrophobic groups from aqueous environment while interacting with the aqueous phase with their hydrophilic groups. Depending upon their bilayer structure and size, liposomes can be categorized as multilamellar, large unilamellar, or small unilamellar. Alternatively, depending upon the driving force for drug release, they can be classified as conventional liposomes, pH sensitive liposomes, cationic liposomes, immuno-liposomes and long circulating liposomes (reviewed in ref (35)). These lipid based particulate carriers can significantly enhance the solubility of poorly water soluble chemopreventives. Different drugs based upon their lipophilic character can distribute either in the phospholipid bilayer, in the interior aqueous phase, or at the bilayer water interface. The lipophilic nature of many chemopreventives including curcumin (table 1.3), resveratrol (65, 66), oryzanol (67), and N-acetyl cysteine (68), make them suitable candidates for liposomal drug delivery where lipophilic core of these liposomes provide an optimum environment for drug entrapment (69).

A liposomal system for the targeted delivery of curcumin (by coating with prostate membrane antigen specific (PSA) antibodies) was also reported to study its partitioning potential (70). It has been observed that DMPC based liposomes possess greater encapsulation efficiency with a more desirable particle size of 100-150 nm as compared to liposomes prepared with dipalmitoyl phosphatidylcholine (DPPC) and egg phosphatidylcholine (PC). Furthermore, DMPC liposomes were found to inhibit (70-80%) cellular proliferation of the human prostate LNCaP and C4-2B cancer cells at 5-10 μM concentration as compared to free curcumin that required 10-fold higher doses to elicit

similar inhibition. Both *in vitro* and *in vivo* studies have shown that liposomal curcumin is much more effective than free curcumin at equimolar concentrations emphasizing that liposomal delivery of curcumin can enhance their uptake and hence bioavailability/activity into the cells (70). A liposomal formulation of curcumin using dimyristoyl-sn-glycero-3-phosphocholine was also tested for its effects on the modulation of signaling pathways involving proliferation, apoptosis and angiogenesis of human pancreatic carcinoma cells (71). When administered at 40 mg/kg (3 times/wk), this liposomal formulation suppressed the growth of BXPC3 and MiaPaCa2 tumors in a xenograft murine model suggesting *in vivo* efficacy of these liposomes (71).

Chemopreventives as liposomal formulations can also be delivered trans-cutaneously through hair follicles (72) providing a reservoir for locally applied substances and to enable topical administration. Jung et al. (72) investigated the penetration depth of a novel class of amphoteric liposomes having iso-electric point at slightly acidic pH to measure the efficiency of trans-follicular delivery of curcumin. They found that these liposomes can penetrate ~35 to 69% of the follicle length depending upon the charge on the liposomes, demonstrating their ability for topical delivery of lipophilic chemopreventives for both therapeutic as well as chemopreventive purposes. However, rapid elimination of these liposomal vesicles by active opsonization is known to limit their overall efficacy which can be avoided by modifying the liposomal surface with polymers such as PEG to confer stealth properties to them. Similar liposomal delivery systems are also reported for active curcumin metabolites like tetrahydrocurcumin (THC). Government Pharmaceutical Organization of Thailand developed a tetrahydrocurcumin cream formulation using phospholipid-derived THC liposomes (73). Dermatological tests for irritation, carried out by Wattanakrai et al. (73) on human female volunteers, demonstrated that these liposomes not only were safe but also possessed a significantly

lower irritation potential compared to the reference material. Furthermore, a corneometer analysis of the skin above antecubital fossae revealed a higher moisturizing effect, which further showed that topical delivery of liposomal curcumin can be used in various skin ailments. However, some of the major problems of this delivery system include stability, poor batch to batch reproducibility, sterilization difficulties, and low drug loading (74).

Table 1.3.

Liposomal formulations of curcumin for parenteral administration.

Formulation	Method	Particle Size (nm)	Encapsulation Efficiency (%)	Advantages	Refs.
DMPC:DMPG:Cholesterol (7:1:8) liposomes of curcumin for parenteral administration.	Vortexing of SMLs	N. R.	N. R.	• Increased curcumin stability in PBS. • Increased efficacy in inhibiting ConA stimulated human lymphocytes and EBV transformed B- cells.	(75)
Pegylated DMPC, Cholesterol, DMPG liposomes of curcumin for parenteral administration.	Extrusion through 0.22 μm filter	N. R.	N. R.	• Significant growth inhibition of Colo205 and LoVo cells in nude xenograft mice.	(71)
DMPC:DMPG (9:1) liposomes of curcumin for parenteral administration.	Extrusion through 0.22 μm filter	N. R.	N. R.	• Significant growth inhibition of CAL27 cells in nude xenograft mice.	(76)
DMPC liposomes coated with PSA specific antobodies.	Sonication of SUVs	100-150	N. R.	• Increased inhibition of LNCaP and C4-2B cells at 10 fold lower doses of curcumin	(70)

N.R: Not reported

2.6. Microemulsions/Microencapsulation

Microemulsions are one of the most widely-used drug delivery systems capable of providing high drug entrapment efficiency with long term stability of hydrophobic molecules (77). These thermodynamically stable, optically isotropic, transparent formulations are characterized by a dynamic microstructure that results spontaneously by mixing lipophilic and hydrophilic excipients in presence of suitable surfactants (78). This microstructure results in high drug solubilization capacity along with free and fast drug diffusion (79) that coupled with lipophilic nature endow them with a high potential for delivering lipophilic compounds like curcumin not only across lipophilic cell membranes but also through skin. Studies by Teichmann et al. (79), demonstrated that curcumin can easily be delivered through the stratum corneum and into the complete follicular infundibula *via* o/w (oil in water) micro-emulsions. These micro-emulsions can be further formulated into hydrogel patches of chitosan or chitosan starch blends to protect the drug from the detrimental effects of pH, light and/or oxygen mediated degradation (80). Once these agents are micro-emulsified and entrapped into a hydrogel like matrix, their stability increases significantly and controlled release at a desirable site can be obtained. Studies have shown that even after 2 months of storage at room temperature mean hydrodynamic diameter of the oily internal phase increases slightly, demonstrating the high stability and efficiency of such hydrogels (80). In addition, the aqueous phase of these emulsions provides hydration to the stratum corneum and moisturizes the skin (80). Drug release from micro-emulsified droplets can be further augmented by using external energy sources such as ultrasonic waves. It has been observed that on application of external energy these droplets undergo a structural reorganization that results in the phase separation of oil droplets from the aqueous vehicle releasing the compound (77). Similarly, a microemsulsion cream formulation of curcumin SLNs was also described by Tiyaboonchai et al. (81). An entrapment efficiency

of 35-70% was demonstrated for curcumin in SLNs with a diffusion mediated controlled release pattern. In addition, the formulation was found to increase the photo stability of curcumin where after 6 months of storage, with no significant change in the viscosity or color of the formulation (81). Although this approach seems promising in enhancing the delivery of potent therapeutics, its usefulness for chemopreventives has not been established in animal and human clinical studies.

Aziz et al. (82) prepared microcapsules of curcumin with gelatin using ethanol/acetone as coacervating agents to separate the two phases that result in precipitation of the drug in spherical microcapsules. They prepared curcumin dispersion in the gelatin solution followed by its addition to ethanol. A Formaldehyde solution (37% v/v) was then added to provide rigidity to gelatin coating. It was reported that micro-encapsulation yield, drug loading and entrapment efficiency all were significantly affected by the solubility of curcumin in the coacervating solvents. They were higher when acetone was used to dissolve curcumin as compared to ethanol in which curcumin tend to disperse at high concentrations used for loading into micro-emulsions. Furthermore, the microcapsules prepared by using acetone were found to possess better flowability and high stability with retention of their spherical shape (82). A similar injectable microparticulate formulation of curcumin using PLGA polymer was prepared and used in breast cancer chemoprevention study (83). These microparticles were found to provide sustained blood and tissue levels for around 1 month by a single subcutaneous injection with tissue levels 10-30 fold higher in brain and lung as compared to that in plasma suggesting their potential to sustain drug levels on subcutaneous administration (table 1.4).

Table 1.4.

Microparticle/microemulsion formulations of curcumin.

Formulation	Method	Particle Size	Encapsulation Efficiency (%)	Advantages	Refs.
Curcumin macrocapsules prepared with gelatin.	Coacervation Method	80-90 μm	75.5	• Improved stability coupled with controlled release.	(82)
PLGA microparticles of curcumin for parenteral administration.	Emulsion Solvent Evaporation	22±9 μm	75	• Sustained plasma curcumin levels for upto a month after a single s/c injection. • Enhanced efficacy as compared to powder curcumin.	(83)
Curcumin microemulsion (o/w) formulated into a hydrogel matrix for topical application.	Surfactant mediated Microemulsification	45-50 nm	N. R.	• Porous polymeric network providing controlled release. • Increases skin hydration.	(80)
Microemulsified curcumin loaded SLNs for topical application.	Microemulsification	450 nm (PDI 0.4)	70	• Increased photostability.	(81)

N.R.: Not reported

2.7. Micelles

Micelles are nanosized colloidal particles formed due to the spontaneous self aggregation (micellization) of amphiphilic polymers in the aqueous phase. Suitable polymeric candidates for micellization are so designed that they have both hydrophobic and hydrophilic segments able to self aggregate in presence of water so as to form a hydrophilic shell and a hydrophobic core. Hydrophilic shell favorably interacts with the surrounding aqueous medium (or with water soluble drugs) and hydrophobic core encapsulate lipophilic drugs (84, 85). This solubilization of different compounds in hydrophobic cores of micelles of amphiphilic copolymers is often dictated by Flory-Huggins interaction parameter. This parameter is a measure of a solute's compatibility with the micelle's core forming component and is inversely related to the drug's solubility. A lower value of Flory-Huggins parameter signifies high compatibility between the solute and the micelle core and hence higher solubility (86). Such drug loaded polymeric micelles are generally thermodynamically stable and are biocompatible formulations that can gradually release the entrapped/adsorbed drug for prolonged period of time at a controlled rate (87, 88). Co-polymerization of PEG with hydrophobic-low molecular weight natural phospholipid compounds like diacyl lipid, fatty acid and bile salts results in such amphiphilic polymers with self aggregation behavior (89). Moreover, since bigger micelles and microparticles are prone to opsonization by reticulo-endothelial system (RES) followed by their clearance through hepatic and splenic endothelial fenestrations (90), presence of non-ionic hydrophilic PEG shell also suppresses the opsonin adsorption and subsequent clearance by the mononuclear phagocyte system. On the other hand presence of lipid moieties, like palmitic acid, in the hydrophobic core results in higher curcumin encapsulation efficiency and presence of ester linkage between PEG and palmitic acid ensures polymer hydrolysis by esterases releasing the entrapped drug inside the cells. Such micelles showed a critical micelle concentration

(CMC) of ~0.12g/L with a particle size of ~50 nm implying their thermodynamically stable nature. However, high drug:polymer ratios required for high encapsulation efficiency is an inherent limitation of such delivery systems (89).

Curcumin micelles using amphiphilic block copolymer of poly(ethylene oxide)-b-poly(ε-caprolactone) with an aim to increase its solubility and stability with controlled delivery has also been reported (91). PCL enable the entrapment of hydrophobic drug in its core and PEO forms the hydrophilic corona of micelles improving the biocompatibility. PEO can also increase the circulation time of these biomaterials by hindering the uptake by the reticulo-endothelial system (91). However, with such a copolymer system, PCL molecular weight determines the encapsulation efficiency and particle size with negligible effect of drug load. Photo and pH stability can also increase drastically due to entrapment of curcumin in polymeric micelles (91). Similar behavior was also observed by Letchford *et al.* with PEG and PCL copolymers. They also showed that CMC was inversely related to the PCL block length and drug solubilization in the core was a function of degree of drug's compatibility with the core forming copolymer (86). Although these studies showed that drug encapsulation depend upon the hydrophobicity of the core forming copolymer, it would be more interesting to see if any correlation exist between the hydrophobicity of the drug and the core forming copolymer block. It might be possible that other drug polymer interactions might also contribute significantly towards the determination of micelle stability, CMC as well as encapsulation efficiency. Tonnesen has shown that in presence of anionic surfactants like sodium dodecyl sulphate, curcumin stability towards pH-mediated degradation (pH 8) increases by 1800-fold due to formation of surfactant micelles and curcumin entrapment inside them (92). However, SDS fails to protect curcumin at very high pH (pH 13) and at such a high pH cationic surfactant like cetyl trimethyl ammonium bromide and dodecyl trimethyl

ammonium bromide afford much higher protection as compared to SDS (93). It has been postulated that at slightly basic conditions (pH 8), negatively- charged head groups of surfactant anions repel $-OH^-$ ions and hence protects curcumin molecules to come in contact with these ions. However, at very high pH (pH 13), curcumin exists as a triply-charged anion (cur^{3+}) which can be effectively entrapped in cationic micelles. Furthermore, due to this high negative charge, it also dissociates from SDS anions and hence is less protected from the deleterious effects of pH (93). However, considering high decomposition rate at pH 7.4 slow release kinetics of curcumin from these nanomicelles limit their ability to deliver therapeutically relevant amounts to the targeted tissues.

Similar micellization behavior of milk casein has also been reported to be effective for improving curcumin's oral administration. Milk has always been used as a preferred delivery system for the curcumin delivery in the traditional medicine for its anti-inflammatory properties. Recent developments have shown that milk casein exist as almost spherical micelles with an average size distribution of <200 nm and can complex hydrophobic drugs like curcumin via hydrophobic interactions. Caseins exist as micellar nanostructures held together by hydrophobic interactions and by calcium phosphate bound at phosphorylated serine residues (94). These casein nanomicelles efficiently bind curcumin molecules in their hydrophobic interior via interactions with their tryptophan and tyrosine residues. However, cell culture studies did not show much difference in the IC_{50} concentration of casein curcumin complex with that of free curcumin (94). But considering the fact that in traditional medicine, turmeric is often given in hot milk, such a delivery system might significantly enhance the bioavailability of such a compound. Recently, inclusion and non-inclusion complexes of drugs with hydroxyl propyl γ cyclodextrin were found to self associate to form nanoscale aggregates

that can solubilize lipophilic drugs like curcumin in a micelle like fashion. These nano-micelles localize to the subcutis, in the hair sheath and in the base of the hair follicles, on tropical application. This delivery system could provide specific delivery of curcumin to the epidermal cells that form the internal root sheath or to the hair follicles for dermal ailments (95).

It is clear that micellization can effectively enhance its solubility as well as stability. However, the main concern with this delivery system is the effect of dilution by the body fluids on parenteral administration. At the reported CMCs for these micelles, it is highly likely that these amphipathic polymer molecules will rapidly disassemble due to dilution on *i.v.* administration, rapidly releasing the whole drug load. Considering curcumin's high rate of metabolism, the injected drug might get eliminated out of the body within hours. To overcome this limitation, it is required that copolymers with very low CMC should be designed that can slowly release the encapsulated drug over longer periods of time.

Although research on most of these delivery systems has demonstrated the potential for increasing bioavailability, two important aspects still need attention: 1) the occurrence of rapid drug metabolism (e.g. for curcumin), which may be mitigated by the application of combination therapies such as with piperine-like enzyme inhibitors; and 2) the need for frequent parenteral dosing, in order to maintain effective therapeutic concentrations in the blood.

Background, Significance and Hypothesis of the Project

Considering the above described potential limitations of curcumin, it appears that this chemopreventive drug needs a "24/7" delivery system like **polymeric implants** that can

continuously release the drug into the systemic circulation without the need for frequent administration. Polymeric implantable drug delivery systems are prepared by homogenous entrapment of drugs in a polymeric matrix and can be effectively used to achieve sustained localized delivery with complete bioavailability into systemic circulation (96). These delivery systems slowly release encapsulated drug directly at the site of implantation to act either locally or to get absorbed into systemic circulation for distribution to other organs. Since these implants are mostly used for local delivery of anticancer drugs, their associated systemic toxicity is significantly minimized with maximizing the drug present at its site of action (97). Furthermore, due to their slow release kinetics, implants can provide drug release ranging from months to years which improves patient compliance (98). These polymeric implants has been found to be effective for many chemotherapeutic drugs and several of such implants are approved by FDA for cancer chemotherapy (table 1.5) (99).

Table 1.5.

FDA-approved implants for cancer chemotherapy (99).

Device	Drug	Polymer	Disease
Decapeptyl	(D-Trp6)LH-RH	PLGA	Prostate Cancer
Lupron Depot	Leuprolide	PLGA	Prostate Cancer
Zoladex	(D-Ser(Bu)6AzGly10-GnRH	PLGA	Prostate Cancer
Gliadel	Carmustine	PCPP-SA	Malignant Glioma

There are 2 type of implantable drug delivery systems, reservoir type and matrix type. In reservoir type implants, drug core is coated by a semi permeable polymeric membrane which controls the rate of drug release and is dependent upon the rate of water influx into the system (Figure 2.6) (99). But these reservoir type implants are often discouraged due to their probability of dose dumping (98). Matrix type implants, on the

other hand, are prepared by homogenously dispersing the drug into the polymeric matrix from where drug gets release into the surrounding medium is mediated by diffusion. These implants are devoid of any dose dumping phenomenon and provide desirable biphasic drug release kinetics (Figure 1.7) (99). This biphasic release consists of a burst release followed by a slow controlled release. Initial burst release delivers the drug for distribution to a large volume, to rapidly reach the therapeutic concentration and a slow, controlled release maintains the therapeutic concentration for a prolonged period of time (Figure 1.8, type (C) release) (100) desirable for natural chemopreventive compounds like curcumin.

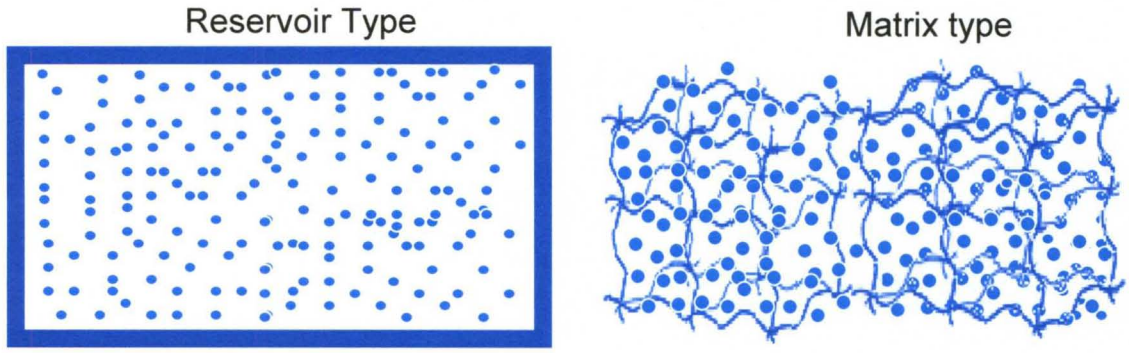


Figure 1.7. Two types of implants used for localized/systemic delivery of chemotherapeutic/contraceptive drugs.

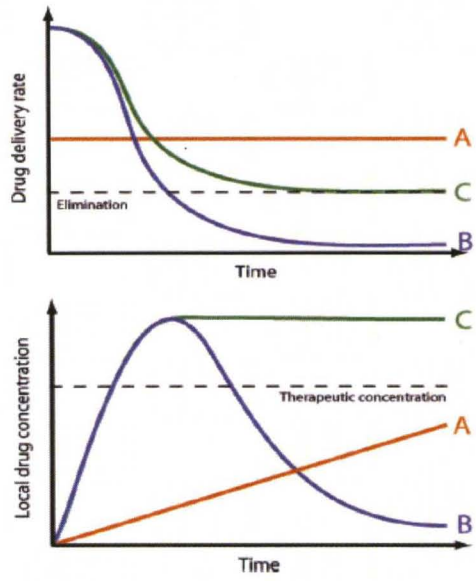


Figure 1.8. Illustrates drug release from 3 theoretical implants. Zero order release (A) from implants may take longer time to reach the therapeutic concentration. A huge burst release (B) from implants may deliver large amounts of drug initially, but may not maintain it at therapeutic levels after some time. A Biphasic release from implants (C) provides an early burst release to rapidly achieve the therapeutic concentrations with a controlled release to maintain therapeutic concentrations (100).

Therefore, we hypothesized that direct systemic administration of curcumin by matrix type polymeric implants can **enhance its chemopreventive efficacy** by (i) **bypassing the oral route** (ii) **eliminating the first pass metabolism** and/or by reducing **the effective doses required** as compared to traditional oral route. To test this hypothesis, we propose following specific aims:

Specific Aim 1. To develop and optimize polymeric implants. Polymeric implants will be prepared by extrusion method using water-insoluble (polycaprolactone) and water-soluble polymers (e.g., F-68, cyclodextrin or poly(ethyleneglycol)) as additives. The drug release kinetics from these implants will be optimized with respect to their drug load, surface area, polymer molecular weight, % of additives to achieve a controlled drug

release. These optimization studies will be carried out under *in vitro* conditions by simulating in vivo extracellular fluid conditions (chapter II).

Specific Aim 2. To study the tissue distribution of curcumin administered by the polymeric implants as well as by the oral route. An HPLC method will be developed and validated for analysis of curcumin in various tissues. This HPLC method will then be used to study curcumin distribution in tissue like plasma, brain and liver with or without the use of tandem mass spectroscopic techniques (chapter III).

Specific Aim 3. To analyze the safety of implants by measuring various inflammatory, proliferative and immune responses. The polymeric implants will be analyzed for their safety in animals by measuring the immunological responses of neutrophils, lymphocytes and macrophages towards implants. Effects on various liver and kidney functions along with changes in various hematological parameters will be evaluated to determine any acute/chronic toxicity associated either with implants or with continuous systemic administration of high doses of curcumin. Pathophysiological studies will also be carried out by histology using H & E staining to determine the intensity of inflammatory reactions at the site of implantation and at adjacent sites (chapter IV).

Specific Aim 4. To determine the chemopreventive/chemotherapeutic efficacy of curcumin delivered via polymeric implants as compared to administered by oral route. Pharmacodynamics of curcumin delivered by these implants as well as the by the traditional oral route will be studied using estrogen-mediated rat mammary tumorigenesis model. Relative efficacy of curcumin treatment by both routes will be determined by analyzing modulation of the various tumor indices (tumor latency, tumor

incidence, tumor multiplicity, and tumor volume) as well as selected molecular targets (chapter V).

Significance of the Project

Data gathered from this research project will provide a drug delivery system that can (i) enhance bioavailability and lower the effective dose of chemopreventive agents compared with dietary route, (ii) allow testing of other agents that cannot be tested for their efficacy under *in vivo* conditions due to their limited quantities; and iii) deliver chemopreventive/chemotherapeutic agents at the target site at controlled doses thus minimizing systemic toxicity. The outcomes will also enable to develop effective strategies for metronomic chemotherapy where low, sustained plasma concentrations (much below their therapeutic concentration) of active compounds are maintained for extended periods of time.

CHAPTER II

DEVELOPMENT AND *IN VITRO-IN VIVO* EVALUATION OF POLYMERIC IMPLANTS FOR CONTINUOUS SYSTEMIC DELIVERY OF CURCUMIN

Rationale

Drug release from implants is a dynamic process that largely depends upon the surrounding environment and polymer characteristics (100). Extracellular fluid composition and volume can drastically alter the rate and extent of drug release (96). Basal rate of drug release from these implants is dependent upon the rate of diffusion of extracellular fluid (ECF)/release media inside the matrix, its tendency to dissolve the drug and efflux of fluid into the surrounding media (100). Other factors like implant surface area, drug load; formulation additives may also contribute significantly to alter the drug release (101, 102) and hence need to be evaluated and optimized for desired release kinetics. Furthermore, depending upon the polymer degradation characteristics, implants can degrade only at the surface (surface erosion type) or degrade slowly in the bulk of the implant (bulk erosion type) to releaser the drug (103). This degradation and release behavior of implants is determined by the hydrolysis rate of polymeric chains and hence polymer selection is the most critical factor in the development of clinically useful implants (103). Poly(ε-caprolactone) (PCL) is an FDA-approved polymer that undergoes bulk erosion with very slow degradation rates and may take months to years to get hydrolyzed (104). Its low melting point (58-60 °C) makes it a suitable candidate to be used for natural chemopreventives which are often thermolabile (105). The focus of

this specific aim, therefore, will be to investigate the influence of the indicated variables on curcumin release from PCL implants under simulated *in vitro* conditions. Results from these studies will provide useful insights regarding the changes in drug release kinetics with changes in drug load, surface area, presence of water-soluble additives and polymer degradation rate. The outcomes will enable us to identify rate determining variables and to alter them to achieve a desirable rate of drug release.

Materials and Methods

Materials

Dichloromethane, poly (ε-caprolactone) 15,000 and 65,000 molecular weight (PCL-15 and PCL-65), 2-hydroxypropyl β-cyclodextrin and phosphate-buffered-saline (PBS) tablets were purchased from Sigma (Sigma-Aldrich, Inc., MO, USA). F-68 was a generous gift from BASF Corporation (Campus Dr., NJ, USA). PEG-8,000 was purchased from Fisher Scientific (New Jersey, USA), ethanol from Pharmco-AAPER (KY, USA) and bovine calf serum (BCS) from Hyclone (Utah, USA). Silastic tubing (3.4 mm external diameter) was purchased from Allied Biomedical (Ventura, CA, USA) and curcumin (>98% purity) was purchased from Across Organics (New Jersey, USA). All the materials were used as received without any further analysis.

Methods

Preparation of Curcumin Implants

Curcumin implants (2, 5, 10 or 20% w/w) were prepared by melt-extrusion technique. Briefly, the polymer (PCL) and curcumin were dissolved in dichloromethane (DCM) and ethanol, respectively and mixed together in the presence or absence of a water-soluble polymer to prepare a homogenous solution of drug and polymer. After evaporation of the solvents at 70 °C on a water bath, the semi-solid residue was dried overnight under vacuum at 65 °C to ensure complete removal of solvents. The dried drug-polymer mixure was filled in 3-ml syringes, heated at 65 °C and molten drug-polymer mixture was extruded through silastic tubing mold attached to the syringe. After few min, the cylindrical implants (3.4 mm external diameter) were removed from the tubing and excised into desired sizes (0.5, 1.0, 1.5 and 2.0 cm) and stored at -20 °C in amber color vials under argon until use.

Curcumin Stability studies

Curcumin (300 µg) was added to 10 ml PBS, PBS supplemented with 10% (v/v) BCS or to 0.7% albumin solution in PBS taken in amber colored vials. The vials were then incubated at 37±0.5 °C in a shaker water bath (Julabo SW23, Germany) shaking at 150 rpm. At different time intervals, 300 µl of solution was aliquoted and analyzed by UV spectrophotometry (Spectramax M2, Molecular Devices, USA) to determine the degradation rate of curcumin in different medias.

Differential Scanning Calorimetry (DSC) Studies

DSC studies were carried out by heating 6-9 mg of each sample from 28 °C to 200 °C at a rate of 10 °C/min (DSC Q20 from TA instruments) in hermetically sealed aluminium pans constantly purged with nitrogen at a flow rate of 50 ml/min. The data was analyzed using Universal Analysis 2000, version 4.5A software.

Powder X-ray Diffraction (PXRD) Studies

PXRD studies were carried out by using Bruker AXS D8 Diffractometer and data was analyzed using XRD commander version 2.6.1 software. The samples were placed onto polymethylmethacrylate (PMMA) sample holder, leveled manually with a clean glass slide and scanned over an angular range of 5° to 55° 2-theta scale with a step time of 0.2 sec.

Analysis of Residual Solvents (DCM) by Gas Chromatography

Since a class II solvent, dichloromethane, was used during the implant formulation; Implants were analyzed for residual DCM content by gas chromatography. Sham Implants (1 cm) were dissolved in 1ml tetrahydrofuran (THF) followed by addition of 3 ml

methanol to precipitate the polymer. Polymer was separated using centrifugation at 10,000 g for 10 min and 1µl of supernatant was injected into the column (DB5MS, 20 m X 0.18 mm) with a split ratio of 70:1. The molecular ion peaks were measured using selective ion monitoring and using THF (molecular ion peak at 31) as the internal standard. The amount of DCM was calculated by using a calibration curve prepared in the similar manner.

In Vitro Drug Release

Release of curcumin from implants (equivalent to 2, 5, 10 or 20 mg/cm in 2, 5, 10 or 20% w/w) was determined under simulated *in vitro* conditions. Briefly, implants were agitated in 5 ml PBS (pH 7.4) supplemented with 10% (v/v) BCS at 37±0.5 °C in a reciprocating shaker water bath (Julabo SW 23, Seelbach, Germany) (150 rpm) and the release medium was changed after every 24 h. PBS was supplemented with BCS (10% v/v) to mimic extracellular fluid composition and to simulate the *in vivo* situation. Albumin present in the BCS binds curcumin *via* vander Waals forces of interaction and provides perfect sink conditions for release of curcumin (106). Curcumin concentration was measured spectrophotometrically at 430 nm against a standard curve following the addition of ethanol (10%, final concentration) to ensure complete dissolution of curcumin.

Scanning Electron Microscopy (SEM)

Surface morphology of implants was studied by SEM using Jeol JSM-5310 (Jeol Ltd, Tokyo, Japan). Sections of the implants before and after the *in vitro/in vivo* release were cut and mounted on a copper base with carbon glue. The sections were sputter coated

with gold using Spi-Module sputter coater (SPI Supplies, West Chester, PA) and analyzed at 25 kV.

Content Uniformity of Implants

Drug contents in implants (n=3) were analyzed to determine the homogeneity of drug distribution and to verify the amount of drug incorporated in each implant. ~~One~~ Nine implants (equivalent to 10 or 20 mg drug/cm implant for 10 or 20% drug load) were weighed and dissolved in 5 ml DCM. Once the implants were dissolved, 5 ml ethanol was added to completely dissolve the drug. The solution was then diluted suitably with ethanol and drug concentration was measured by UV spectrophotometer at 430 nm (Spectramax M2, Molecular Devices, USA).

Residual drug in the implants recovered from animals was also measured similarly and the average rate of release was calculated as follows:

$$\textbf{Average Daily Release} = \frac{\text{Initial amount - Residual amount}}{\text{Time (days)}}$$

Stability Studies

Implants (1 cm) with 20% drug load were prepared using the standardized procedure and individually stored in amber colored vials. The vials were sealed by parafilm and stored in dark at 4, 25 and 40 °C for a period of 1, 3, 6 and 12 months. After specific time intervals, implants were removed and analyzed for curcumin content as described in content uniformity section. The implants were also studied for changes in their release kinetics after storage at above temperatures for 4 months.

Results and Discussion

Stability of Curcumin in Different Media

Curcumin is highly unstable at physiological pH (pH 7.4) and instability increases further with pH (107). However, it is known that presence of plasma proteins increases curcumin's stability and increases its half-life from ~30 min to ~8 h (106, 107). Therefore, we determined the stability of curcumin in presence and absence of albumin as well as in simulated extracellular fluid (ECF) conditions using PBS supplemented with 10% (v/v) BCS (Figure 2.1). It was found that curcumin exhibits first-order degradation kinetics where rate of curcumin degradation was proportional to its concentration in the solution. Curcumin was highly unstable in PBS and ~90% drug degraded within 2 h (107). The presence of serum and albumin were found to increase the stability of curcumin substantially. The half-life increased from few min to 39 h with BCS and to 131 h with albumin. These results suggest that albumin and other components bind curcumin and hence stabilize it towards pH-mediated degradation (106).

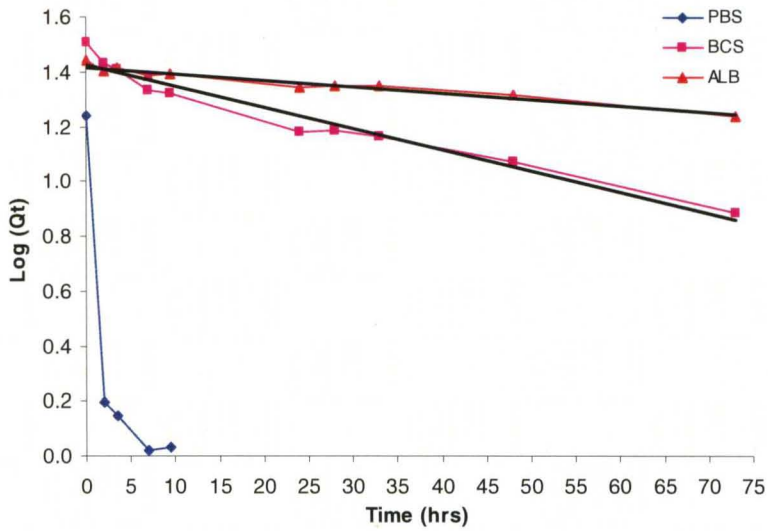


Figure 2.1. Degradation behavior of curcumin in phosphate-buffered saline (PBS), PBS supplemented with 10% bovine calf serum (BCS) and 0.7% albumin solution (n=3, SD<5). The ordinate axis represents logarithm of drug concentration after specific time intervals.

Differential Scanning Calorimetry (DSC) Studies

To check the thermal stability of curcumin and polymer, both the components alone as well as in combination were analyzed by DSC. Curcumin and polymer both were found to be stable by differential scanning calorimetry (Figure 2.2). Melting temperature (Tm) of PCL-65 was found to be 60-61 °C (104) and that of curcumin at 177 °C (108). However, drug-polymer blend showed presence of only one melting endotherm at 58 °C corresponding to PCL polymer and no melting endotherm corresponding to curcumin was observed at 10% drug load (10 mg/cm). The absence of curcumin melting endotherm by DSC in the implants revealed that the drug was dispersed at a molecular level in the polymer matrix at 10% w/w composition and was stabilized in its amorphous form by the polymer (109). However, slight crystallization was observed at 20% w/w drug load with a broad melting endotherm suggestive of slight curcumin crystallization at higher drug loads.

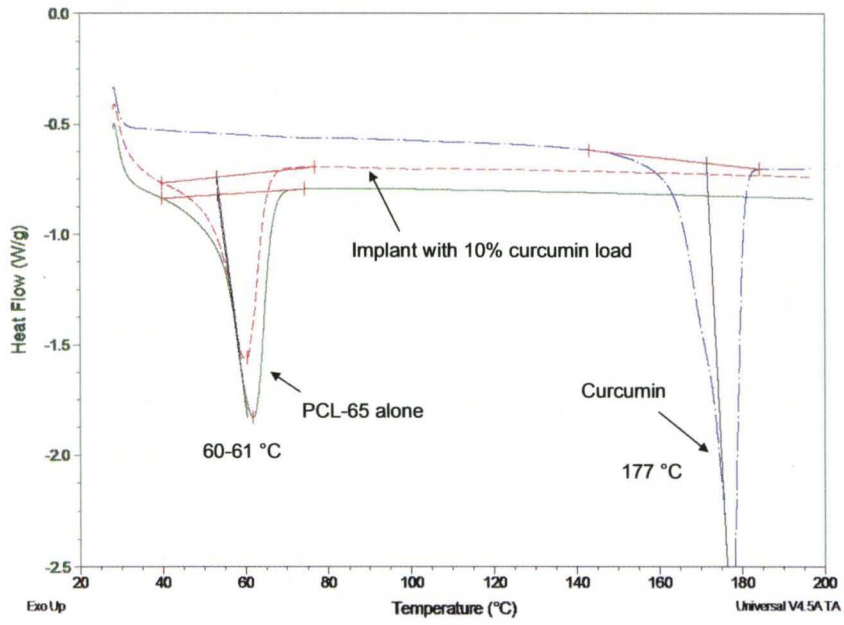


Figure 2.2. DSC thermograms of PCL-65, curcumin and their implants. These studies were carried out by heating each sample (6-9 mg) from 28 °C to 200 °C at a rate of 10 °C under constant nitrogen purge.

Powder X-ray Diffraction (PXRD) Studies

PXRD studies confirmed the semi-crystalline nature of PCL with presence of 2 characteristic peaks at 23° and 25.5° (Figure 2.3). Curcumin on the other hand revealed a crystalline structure with presence of number of characteristic peaks at 17.5, 18.5, 21.5, 25, 26 and 28° (110). Implants containing 10% (w/w) curcumin in PCL, however; showed a halo pattern with presence of only 2 peaks characteristic for PCL. These results further confirmed the amorphization of curcumin at 10% (w/w) drug loading in implants.

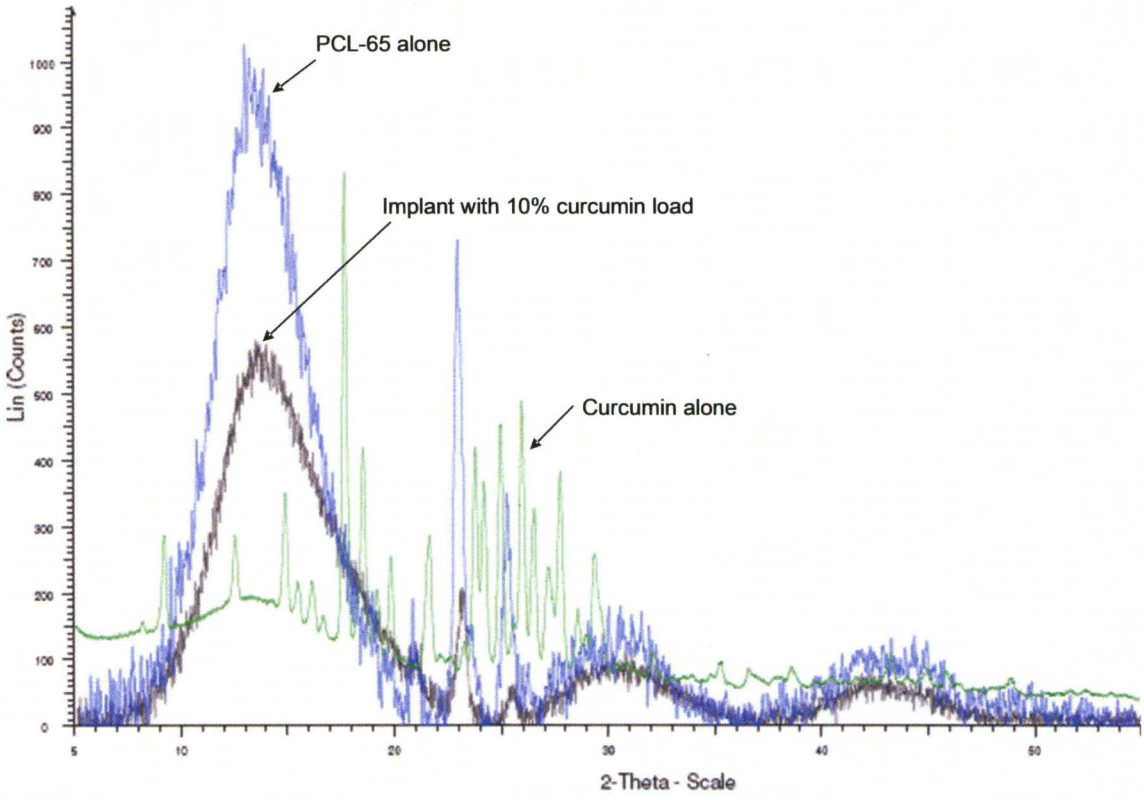


Figure 2.3. Powder X-ray diffractographs of PCL, curcumin and implants containing 10% (w/w) curcumin. The samples were placed onto polymethylmethacrylate (PMMA) sample holder, leveled manually with a clean glass slide and scanned over an angular range of 5° to 55° 2-theta scale with a step time of 0.2 sec.

Drug Content Analysis

To ensure the homogenous distribution and batch to batch reproducibility of curcumin in the polymer matrix, 1-cm implants (10 mg drug/cm implant) were dissolved in DCM:ethanol (1:1), suitably diluted and were measured for curcumin. Analysis of drug content in implants showed that the implants were uniform with homogenous dispersion of drug. Almost 95% ± 1.2% drug was recovered from PCL implants prepared in the same batch or different batches, with negligible variability in drug loading between the implants prepared at different times. This showed that curcumin implants prepared under these conditions were reproducible with respect to drug content and drug distribution.

In Vitro Drug Release: Effect of Polymer Molecular Weight

Since molecular weight of polymers is one of the determining factors for rate of influx and efflux of extracellular fluid through the polymeric matrix (111), different implants were prepared by blending PCL of 15,000 (PCL-15) and 65,000 (PCL-65) molecular weight in different proportions with 10% drug load (10 mg/cm). The implants were then analyzed for their daily drug release for up to ~140 days (Figure 2.4). Data showed that initially ~17% drug was released from PCL-15 implants in one week as compared to around 13% from PCL-65 implants and decreased slightly with increasing concentration of PCL-65. Daily drug release was slightly higher from PCL-15 implants during first week as compared to PCL-65 after which it was similar from both the implants up to 60 days. However, after ~60 days drug release was higher from PCL-65 implants as compared to PCL-15. Daily release from PCL-65 implants was ~3 times higher (30-35 μg/day) as compared to PCL-15 implants (10-12 μg/day) after 100 days. It appears that the pore size of the polymeric matrix of both PCL-15 and PCL-65 is greater than the molecular size of the curcumin molecules and hence is not the limiting factor for the drug diffusion

during initial period. However, with time when the path length of solvent diffusion increases PCL-65 offers least resistance to the drug release and hence the drop in daily drug release is minimal with PCL-65 implants (112).

This study also showed that drug release from these polymeric implants follow biphasic release kinetics. A burst release was observed initially which declined rapidly during the first 25 days followed by a much slower decline for next 100 days. Two hundred to 250 µg curcumin was found to be released on day 1 which then declined with time and dropped to <100 µg/day after 20 days. This burst release could be due to release of surface-bound drug, followed by a more controlled release from the inner layers of implant's polymer matrix. Such a burst effect can be useful in cases where rapid achievement of plasma concentration of therapeutic agents is desired which can then be maintained with slow controlled release of drugs (100). However, in cases where such a burst release is undesirable, these implants can be incubated in ethanol for 1-2 h to get rid of most of the surface bound drug. Furthermore, the decline in drug release during the diffusion from the inner layers of polymeric matrix could be attributed to either reduction in drug's permeability due to increased crystallinity of polymer and/or due to a decrease in concentration gradient of drug in outer layers of polymer matrix (111). It is known that due to semi-crystalline nature of PCL, its crystallinity increases with time in presence of aqueous release media which might result in increased resistance for diffusion of aqueous fluids and hence decreasing the drug release with time (102). Moreover, a decrease in concentration gradient of drug in outer polymer layers result in longer path length for diffusion of aqueous fluid into deeper layers which also result in a decrease in drug release (102). However, when cumulative release data (Figure 2.4 inset) from these implants was analyzed by zero order, first order, Higuchi and Hixson Crowell models for drug-release kinetics (113), it was found that drug release from PCL

implants followed diffusion-mediated Higuchi kinetics (r^2 > 0.98). It shows that PCL implants are able to release drug at a controlled rate over an extended period of time and the drop in drug release was indeed partly due to longer path length of diffusion (113).

The implants were also analyzed by SEM before and after the *in vitro* release. The freshly-prepared implants were found to be smooth with homogenous dispersion of drug without any visible evidence of discontinuities. No significant difference was observed in the polymeric matrix 140 days after the *in vitro* release which showed that the polymeric matrix was stable in the release medium for at least this period of time. However, small amount of drug crystallization was observed in these implants which might be due to slow crystallization of amorphous drug in the polymer matrix that can occur when it comes in contact with the aqueous release media (Figure 2.5a-c).

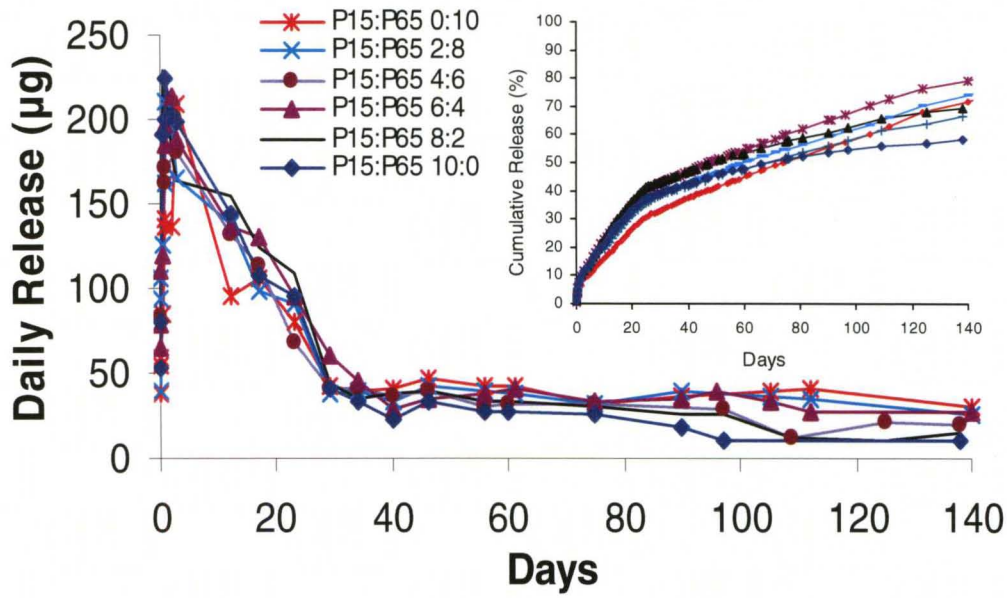


Figure 2.4. Effect of polymer composition on drug release. PCL-15 and PCL-65 were mixed together in different factions to prepare implants and drug release was determined. Data presented denote average of three replicates. SD, generally 5-10% has been excluded for clarity.

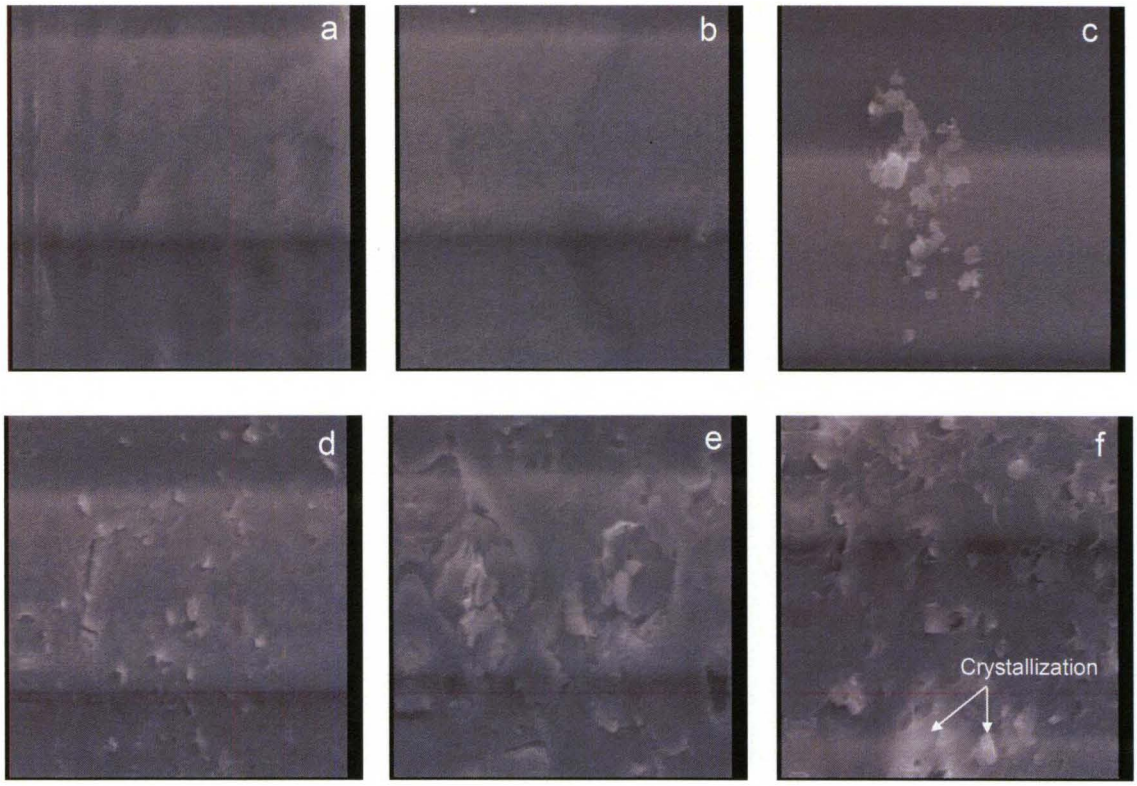


Figure 2.5. Scanning electron microscopy pictures (1,000 times magnification) of (a) sham implant (b) implant with 10% drug load, (c) implant with 10% drug load after 140 days of release under *in vitro* conditions, (d) Implants with 20% drug load, (e) implants with 30% drug load and (f) Implants with 20% drug load showing traces of crystallization (2,000 times magnification).

Effect of Drug Loading

These implants were also analyzed for the effect of drug loading on daily drug release. It has been found that due to channeling effect, increasing drug loads lead to higher drug release (114). Therefore, implants with 2%, 5%, 10%, 20%, 30%, 40% and 50% drug load (2, 5, 10, 20, 30, 40 and 50 mg per cm implant, respectively) were prepared and analyzed for daily drug release (Figures 2.6 and 2.7). It was found that drug release was proportional to drug concentration for only up to 10% drug load and no further increase in the release was observed with higher drug loads. Daily drug release was ~2-3 times higher from 5% implants as compared to 2% implants and similarly was 1.7-1.9 times higher from 10% drug load implants as compared to 5% drug load at all-time points. It

appears that only up to 10% drug load, the channeling effect increases, enhancing the porosity of matrix for diffusion of release media. But, at higher drug loads, solubility of curcumin in the polymeric matrix becomes a limiting factor. Furthermore, due to lipophilic nature of curcumin, increasing drug concentration lead to an increase in lipophilicity of the curcumin-polymer matrix which could also be a contributing factor leading to retardation of influx of release medium (112). SEM analysis of these implants showed that at drug loads >10%, degree of discontinuities and extent of drug crystallization in the polymeric matrix also increased proportionately (Figure 2.5d, e and f) which could also be a limiting factor.

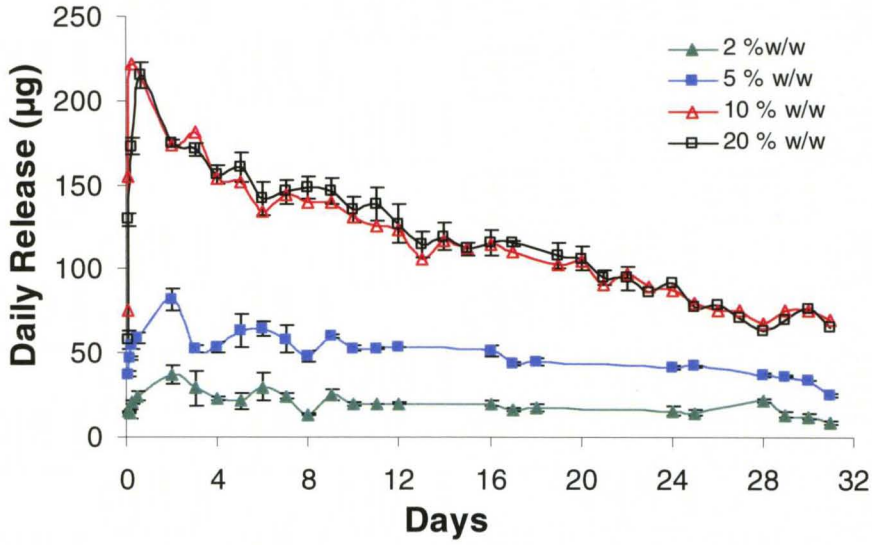


Figure 2.6. Effect of drug loading (2, 5, 10 and 20% w/w drug load) on daily drug release. Data presented denote average of three replicates. SD, generally 5-10% has been excluded for clarity.

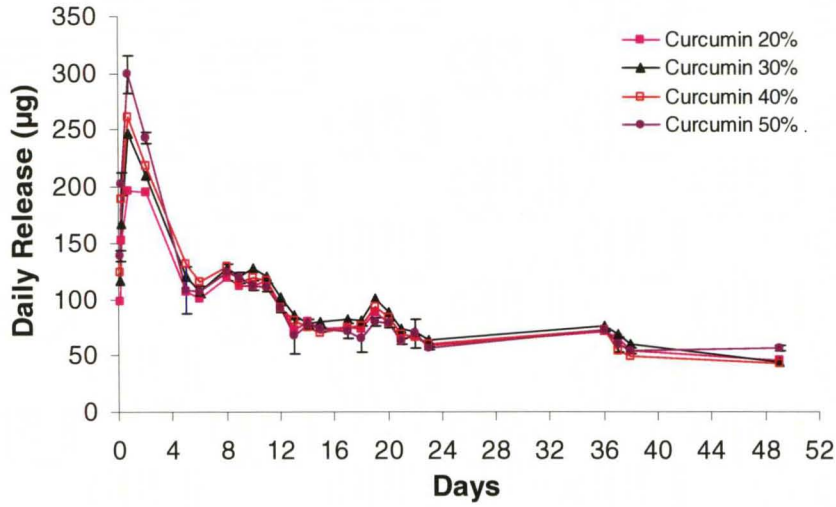


Figure 2.7. Effect of drug loading (20, 30, 40 and 50% w/w drug load) on daily drug release. Data presented denote average of three replicates. SD, generally 5-10% has been excluded for clarity.

Effect of Surface Area

Since the drug release from implants was found to follow diffusion mediated 'Higuchi kinetics, it was expected that an increase in surface area should proportionately increase the drug release. Therefore, to study the effect of surface area, implants of different sizes were prepared: 0.5 cm with a surface area of 0.69 cm^2 (10 mg drug), 1 cm with a surface area of 1.21 cm^2 (20 mg drug) and 2 cm with a surface area of 2.24 cm^2 (40 mg drug); and analyzed for daily drug release (Figure 2.8). As expected, a proportionate increase in drug release was observed with increasing surface area. Daily drug release from 1 cm implants was ~1.6 to 1.8 times higher as compared to 0.5 cm implants after 1 week. Similarly, daily drug release from 2 cm implants was 1.3 to 1.5 times higher as compared to 1 cm implants. Since drug release from these implants was diffusion mediated, according to Fick's law of diffusion as the size of the implants increases, the effective surface area for diffusion of extracellular fluid increases, which in turn, increases the amount of drug efflux from the polymeric matrix (115). Therefore, in order to have maximum drug release, 2-cm implants were selected for further studies.

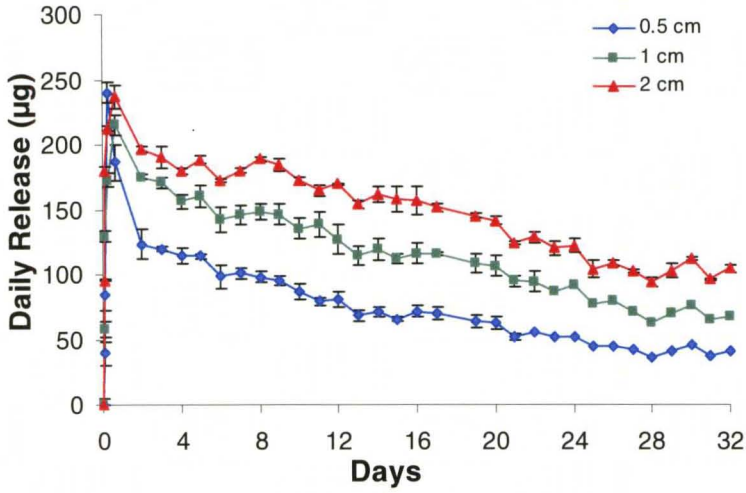


Figure 2.8. Effect of surface Area (implant size) on daily drug release. Data presented denote average of three replicates. SD, generally 5-10% has been excluded for clarity.

Effect of Formulation Additives

Addition of certain excipients in the polymeric matrix influences drug release significantly. They may increase or decrease the drug release rates by changing the matrix lipophilicity, matrix porosity, microclimate pH and or polymer drug interactions (101, 116). Excipients, especially, hydrophilic polymers compatible with the polymeric matrix are helpful in providing aqueous channels to facilitate the diffusion mediated drug release. Due to their water solubility, such hydrophilic excipients preferentially dissolve in the ECF and form a porous matrix enhancing the drug release (117). This effect is more profound in case of less permeable polymeric matrices, especially where drug release is dictated by diffusion through the polymer. Furthermore, a reduction in implant's rigidity and viscosity due to a relative decrease in matrix forming polymer content (due to addition of water soluble polymers) might also be a contributing factor towards enhancement of drug release.

Therefore, we studied the effect of several water-soluble polymeric additives for their influence on drug release. Implants were prepared using PCL-65, 20% drug load and 10% of either F-68, PEG-8,000 or 2-hydroxypropyl β-cyclodextrin (HPCD) and the drug release was compared with the control implants prepared without any such additives (Figure 2.9). It was observed that the presence of 10% F-68 or PEG decreased the drug release to some extent as compared to control implants. Implants prepared without any additive or with HPCD showed 12% drug release over a period of 31 days as compared to 9.5% drug release from implants prepared with F-68 or 11% with PEG. However, when PEG concentration was increased to 35% (w/w), a much higher burst release was observed with higher release for around first 20 days as compared to implants with 10% F-68 (Figure 2.10). Furthermore, since higher concentrations of PEG are expected to decrease the half-life of degradation of polymeric matrix, all implants used for *in vivo* studies were prepared with 35% (w/w) PEG as a formulation additive.

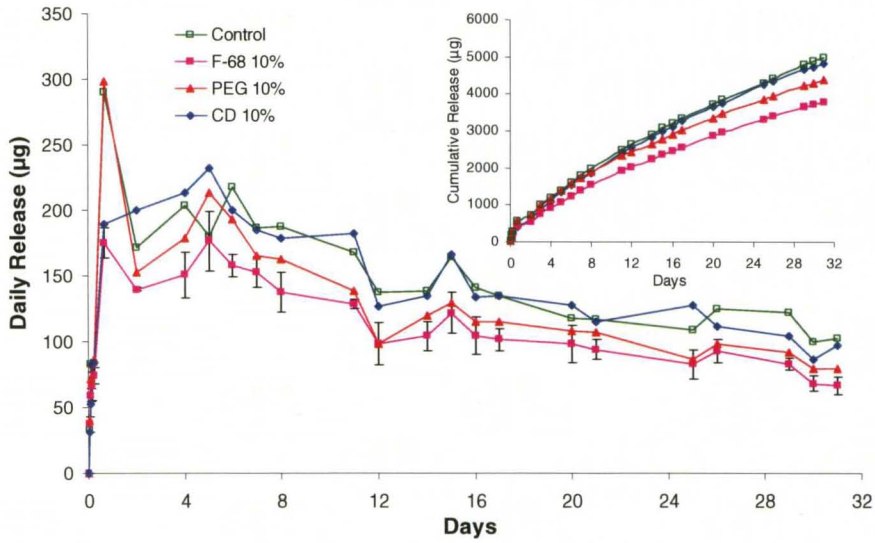


Figure 2.9. Effect of water soluble additives on daily drug release. Data presented denote average of three replicates. SD, generally 5-10% has been excluded for clarity and is shown only for one set of implants.

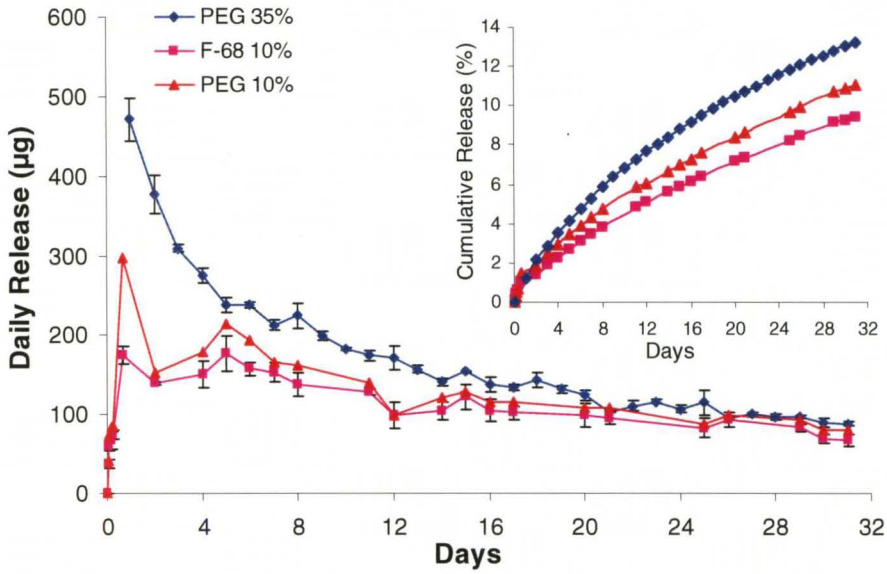


Figure 2.10. Effect of 35% (w/w) PEG of 8000 molecular weight (PEG) on daily drug release as compared with 10% (w/w) F-68 or with PEG. Data presented denote average of three replicates.

Effect of Porogens/salts

It is known that certain salts like ammonium bicarbonate ($(NH_4)_2CO_3$) helps in forming a porous matrix by releasing CO_2 on coming in contact with water (118). Therefore, we prepared P-65 implants with 20% drug load, 10% F-68 and 20% of either $(NH_4)_2CO_3$, citric acid or NaCl. However, it was observed that $(NH_4)_2CO_3$ released CO_2 even while heating during initial stages of implant preparation which was undesirable. On the other hand, implants with NaCl and citric acid did not show any significant increase in drug release (Figure 2.11). Furthermore, increasing the NaCl concentration from 20 to 50% also did not enhance the drug release (Figure 2.12). Therefore, salts were excluded from the formulation.

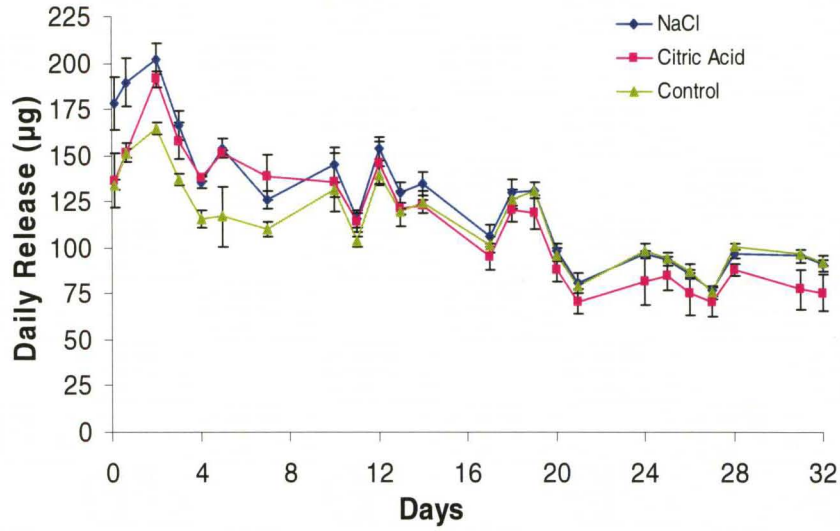


Figure 2.11. Daily drug release from implants prepared with NaCl or citric acid (20% (w/w)) compared with control implants with no salts. Data presented denote average of three replicates.

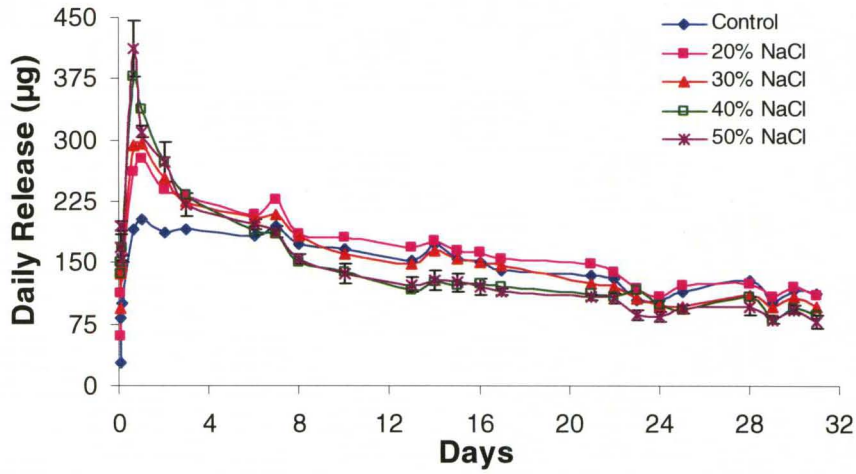


Figure 2.12. Daily drug release from implants prepared with different concentrations of NaCl (20, 30, 40 and 50% (w/w)) compared with control implants prepared without any NaCl. Data presented denote average of three replicates. SD, generally 5-10% has been excluded for clarity and is shown only for one set of implants.

Effect of Storage on Drug Release and on Stability of Entrapped Drug

Since we found that curcumin was dispersed in the polymeric matrix in amorphous form it was expected that with time amorphous curcumin might tend to revert back to its more stable crystalline form during storage. Since, amorphization of curcumin was one of the

reasons that increased its solubility in the release medium, we wanted to study if storage of implants affects the daily drug release. Therefore, 1-cm implants with 20% w/w drug load (20 mg drug/cm implant) were prepared and stored in dark at 4, 25 and 40 °C, to determine the effect of storage and of different temperature conditions on daily drug release (Figure 2.13). As evident from the figure, daily drug release profile was unaltered with almost similar amounts of drug being released from all the implants stored at different temperatures for 4 months, suggestive of stability of these implants with respect to drug release for a period of at least 4 months.

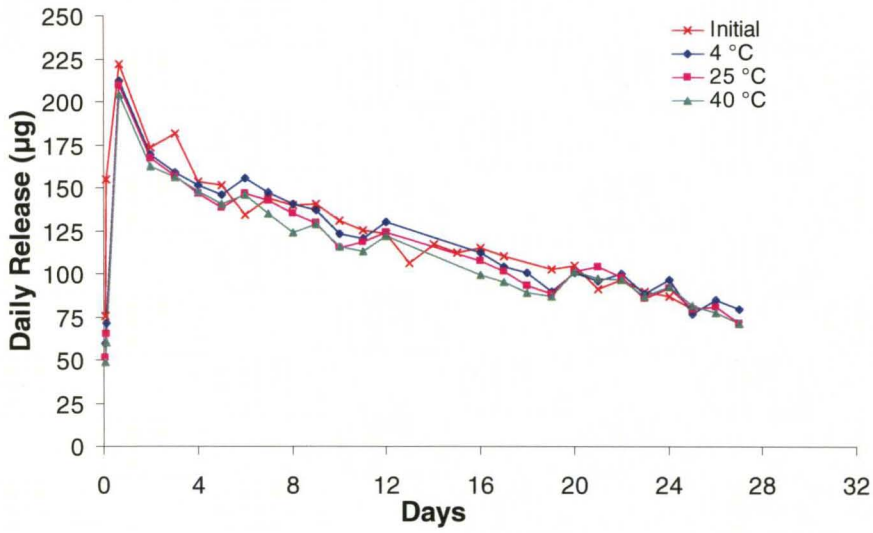


Figure 2.13. Implants were stored for 4 months at different temperatures (4, 25 and 40 °C) to determine the effect of storage and temperature on daily drug release. Data presented denote average of three replicates. SD, generally 5-10% has been excluded for clarity.

Furthermore, the effect of storage and temperature was also studied on entrapped curcumin content by measuring the amount of curcumin in implants. As is shown in table 2.1, almost similar levels were observed in all implants stored at 4, 25 and 40 °C for 1, 3, 6 and 12 months suggestive of curcumin's stability in the polymeric matrix for at least 1 year.

Table 2.1.

Effect of storage on curcumin content in implants stored at different temperatures for a period of 1 year.

	Curcumin Content					
	4 °C		25 °C		40 °C	
Time (Months)	Mean (mg)	SD	Mean (mg)	SD	Mean (mg)	SD
0	17.88	0.2				
1	16.62	0.3	17.7	0.4	16.9	0.4
3	18.34	0.2	18.44	0.4	18.94	0.2
6	17.5	0.1	16.68	0.5	16.14	0.3
12	17.3	0.1	17.23	0.2	17.71	0.1

Conclusions

In this study we developed and optimized polymeric implants of curcumin with an aim to bypass the oral route and to enhance its efficacy by continuously delivering curcumin at a controlled rate, directly into the systemic circulation. Curcumin due to its lipophilic nature was found to be miscible with the polymer and showed a diffusion-mediated biphasic release pattern in accordance with Higuchi kinetics. Drug release was found to increase with increasing surface area, drug loads for up to 10% (w/w) and with PEG-8k at 35% w/w composition. Furthermore, different porogens and water soluble excipients like F-68, HPCD and PEG-8K at 10% (w/w) load were found to be ineffective. These *in vitro* studies showed that polymeric implants could be a viable alternative for the delivery of potent chemopreventives like curcumin and afford a continuous ("24/7")-delivery system that needs to be further optimized under *in vivo* conditions.

CHAPTER III

TISSUE DISTRIBUTION OF CURCUMIN FROM POLYMERIC IMPLANTS AS COMPARED TO ORAL ADMINISTRATION

Rationale

In vivo drug release often does not correspond to *in vitro* drug release due to the presence of endogenous enzymes, lipids and other constituents in extracellular fluid (ECF) (100). Furthermore, presence of perfect sink conditions in tissue environment (which are often difficult to achieve under *in vitro* conditions) also increases the drug release. This dynamic nature of tissue environment exerts a significant influence on efficacy of such polymeric implants (100). Therefore, focus of this section was to investigate *in vivo* release from polymeric implants after subcutaneous (s/c) implantation. Drug distribution in tissues would provide evidence regarding the release and distribution of drug in various tissues through systemic circulation as well as at local site of implantation. Therefore, drug concentration was measured in highly perfused tissues including plasma, liver and brain to determine drug concentration achievable through systemic distribution. Furthermore, modulation of hepatic xenobiotic metabolizing enzymes was also studied as a preliminary measure of chemopreventive efficacy of curcumin (8, 13) delivered *via* implants.

Materials and Methods

Materials

Medical grade poly (ε-caprolactone) of 121,000 molecular weight (PCL) was purchased from SurModics Pharmaceuticals (Birmingham, AL). Dichloromethane (DCM), acetonitrile, anhydrous citric acid and phosphate-buffered-saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO), polyethylene glycol of 8,000 molecular weight (PEG) from Fisher Scientific (Fair Lawn, New Jersey), ethanol from Pharmco-AAPER (Louisville, KY). Bovine calf serum (BCS) was purchased from Hyclone (Logan, UT) and silastic tubing (3.4 mm internal diameter) from Allied Biomedical (Ventura, CA). Curcumin (C-3 complex) extracted under GMP (good manufacturing practices) was a generous gift from Sabinsa Corporation (East Windsor, NJ). All the materials were used as received without any further analysis.

Methods

Preparation of Curcumin Implants

Curcumin implants were prepared by following the procedure described in chapter 2. Briefly, curcumin (20% w/w) was dissolved in ethanol and polymers (PCL and PEG in 65:35 ratio) were dissolved in DCM. Both solutions were then mixed together to prepare a homogenous solution of drug and polymers. The solvents were evaporated on a water bath maintained at 65 °C, followed by overnight drying under vacuum at 65 °C to remove residual solvents to prepare an amorphous molecular dispersion of drug in polymer. The dried molten polymer was then extruded through silastic tubing mold attached to a syringe. After few min, the cylindrical implants (external diameter 3.4 mm) were removed from the tubing and excised into desired lengths (2 cm, 200 mg containing 40 mg drug) and stored at -20 °C under argon until used.

In Vivo Studies

In vivo cumulative release was studied in female ACI (Augustus Copenhagen Irish) rats following an approved protocol from the Institutional Animal Care and Use Committee (IACUC). Animals were provided with purified diet; AIN-93M and purified water *ad libitum* until termination of the study. Two 2-cm implants containing 20% (w/w) curcumin (20 mg/cm) were subcutaneously grafted at the back of the 7-8 week-old female rats as described previously (119). Animals were euthanized at different time intervals by asphyxiation and blood, liver, brain, local tissues and implants were collected. Implants were dried overnight under vacuum, weighed and stored at -80 °C until analysis of the residual curcumin content.

Residual Drug Determination/Cumulative Drug Release

Initial and residual curcumin content in implants (n=3) was analyzed to determine the cumulative drug release. Implants recovered from animals were dried over-night under vacuum and dissolved in 5 ml DCM. Once the implants were dissolved, 5 ml ethanol was added to completely dissolve curcumin. The solution was then diluted suitably (1:10) with ethanol containing 20% DCM followed by in ethanol (1 in 40). Drug concentration was measured by UV spectrophotometer at 430 nm to calculate the amount of curcumin released *in vivo* using following equation:

$$\textbf{Average Daily Release} = \frac{\text{Initial amount - Residual amount}}{\text{Time (days)}}$$

Analysis of Plasma and Tissue Curcumin Levels

Plasma pooled from all the animals (1.5 ml) was mixed with 200 µl of 0.5 M sodium acetate to reduce the pH to 5. Plasma was then extracted thrice with 3 ml ethyl acetate.

Ethyl acetate extracts were pooled and dried under vacuum. The dried residue was reconstituted in 100 µl of acetonitrile (ACN), one half of the solution was analyzed by HPLC to determine the plasma concentration. Similarly, liver or brain tissue (~500 mg) from each animal was homogenized in 3 ml PBS (pH 7.4) containing 200 µl of 0.5 M sodium acetate. The homogenate was then extracted twice with 2 volumes of ethyl acetate. After evaporation of ethyl acetate under vacuum, the residue was reconstituted in 1 ml ACN. The solution was filtered through 0.45 µ glass-microfiber filter and evaporated again under vacuum. The residue was finally reconstituted in 100 µl ACN and one half of it was analyzed by HPLC using Shimadzu liquid chromatography system equipped with LC-10ADVP pump, RF-10AXL fluorescence detector and a Shimadzu C_{18} column of 5 µm particles (250 x 4.6 mm). The three curcuminoids were separated by using ACN and 1% citric acid (adjusted to pH 2.5) at a flow rate of 1 ml/min with a gradient elution in which ACN concentration was increased from 0 to 30% in first 5 min, followed by an increase to 45% in next 5 to 20 min. ACN was then maintained at this ratio till 36 min. Curcuminoids were detected using 410 and 500 nm as excitation and emission maxima, respectively, in the fluorescence detector.

Microsome Extraction

Liver (~100 mg) was homogenized in 0.25 M sucrose buffer (pH 7.4) at 3,000 rpm with Polytron. The homogenate was centrifuged at 3,000 g for 20 min at 4 °C to separate the nuclear content. The supernatant was collected and again centrifuged at 11,000 g for 20 min at 4 °C to separate the mitochondria. The post-mitochondrial supernatant was then transferred to ultracentrifuge tubes and finally centrifuged at 100,000g for 1 h at 4 °C to separate the microsomes. The pellet was suspended in 1 ml sucrose buffer, aliquoted and stored at -80 °C until use.

Analysis of Cytochrome P450s and GSTμ (GSTM)

Microsomal proteins were quantified using bicinchoninic acid (BCA) method (120) using BCA™ Protein Assay kit (Thermo Scientific, Rockford, IL) and were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel. Protein bands were then transferred to polyvinylidene diflouride (PVDF) membrane which was incubated with 5% non-fat dried milk in Tris-buffered-saline for 1 h at room temperature (25 °C) to block the non-specific binding sites. The membrane was then incubated with primary antibodies for CYP1A1 and GSTM, followed by horseradish peroxidase conjugated secondary antibody. The bands were detected by enhanced chemiluminescence using Pierce® ECL Western-Blotting Substrate (Thermo Scientific, Rockford, IL) and quantified using VersaDoc Imaging System (BioRad Laboratories, Hercules, CA).

Determination of CYP3A4 Activity

CYP3A4 activity was measured using P450-Glo™ CYP3A4 assay with Luciferin-IPA (Promega Corporation, Madison, WI) following manufacturer's protocol by replacing NADPH regenerating system with NADPH (5 mM).

Statistics

All groups were compared by students T test for statistical significance. Blank implants and curcumin diet groups were compared with untreated controls and curcumin implants were compared with blank implants for all statistical purposes at a significance level of p value <0.05 ($n=3$-4).

Results and Discussion

This specific aim was designed (i) to analyze the *in vivo* release of curcumin from polymeric implants, (ii) to derive an *in vitro-in vivo* correlation of drug release, (iii) to compare the tissue distribution of curcumin delivered *via* these implants with curcumin delivered *via* diet and finally (iv) to compare preliminary efficacy of curcumin delivered *via* both routes by measuring modulation of hepatic cytochrome levels. Therefore, to achieve these objectives, various *in vivo* release studies were done by subcutaneously implanting two 2-cm implants (10% or 20% w/w drug load either with F-68 (10%) or PEG (35%)) beneath the skin of female ACI rats and were recovered after predetermined time intervals by sacrificing the animals.

In Vivo Drug Release Studies

Effect of Drug Load

To study the effect of curcumin drug load on *in vivo* release, implants with 10 and 20% drug loads were implanted at the back of female ACI rats and were recovered after specific time intervals to measure the residual drug load, shown in Figure 3.1. The drug release was found to be diffusion mediated and increased with drug loading as observed under *in vitro* conditions. These studies showed that initially ~377±52 µg/day drug was released over a period of 1 week with 10% drug load which dropped to ~292±59 µg/day over a period of 3 weeks. However, from implants with 20% drug load, ~666±160 µg/day was released initially which reduced to ~294±110 µg/day after 3 weeks. The drug release was ~1.7 times higher during first week from 20% drug load implants as compared to 10% drug load (Figure 3.2). Since initial burst release is from the surface adsorbed drug which is proportional to the drug load, it was higher with high drug load implants (111). However, as the drug release started from inner polymeric layers;

diffusion mediated and drug load independent release was observed as was found under *in vitro* release studies (111).

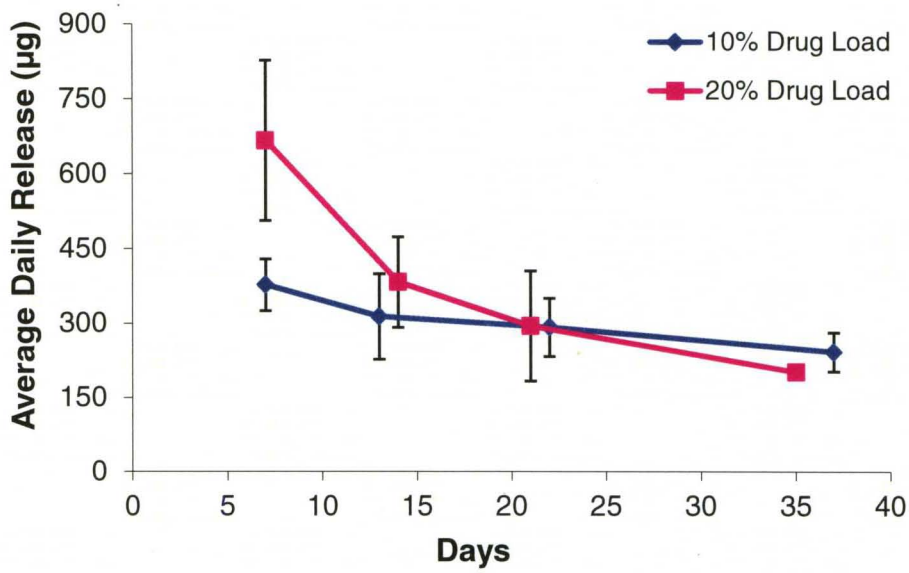


Figure 3.1. Average daily *in vivo* drug release from 2-cm implants with 10 and 20% drug load.

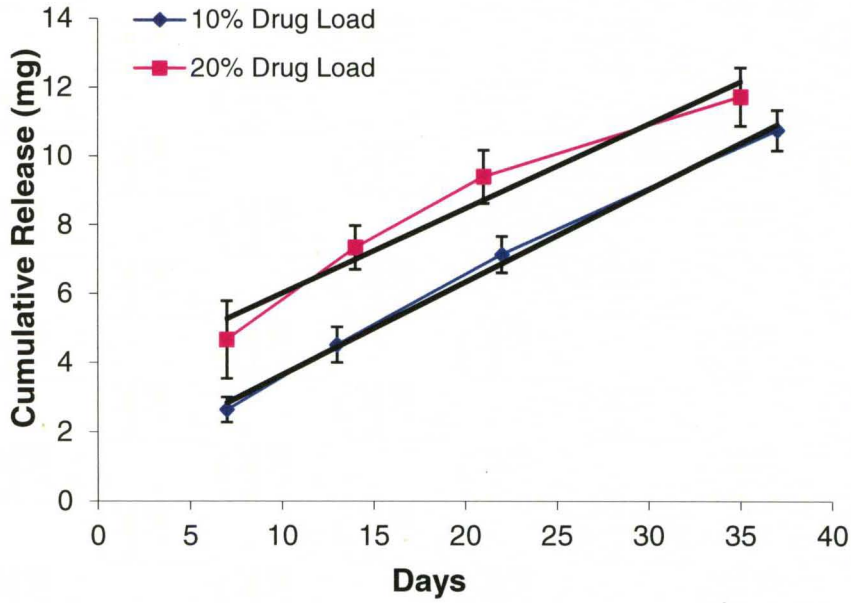


Figure 3.2. Cumulative *in vivo* drug release from 2-cm implants with 10 and 20% drug load.

Effect of Water Soluble Additives

It was observed under *in vitro* conditions that presence of PEG (35% w/w) increases the drug release as compared to that of F-68 (10% w/w). Therefore, to determine the effect of these water soluble additives, *in vivo* drug release was also studied from implants containing 20% drug load prepared either with 10% F-68 or with 35% PEG. *In vivo* release from F-68 implants was studied for a period of 16 months (70 weeks) (table 3.1) and from PEG for 6 months (table 3.2). *In vivo* release was found to reveal similar pattern as was observed under *in vitro* conditions with slightly higher release from PEG (35%, w/w) implants as compared to F-68 (10% w/w) implants both during initial burst phase as well as during 2nd controlled release phase. It appears that PEG being highly water soluble not only increases the hydrophilicity of the polymeric matrix but also provides a more porous microstructure that results in enhanced water permeation into the matrix (117) and hence increased release at such high PEG levels (35% w/w).

Furthermore, *in vivo* release was observed to mimic the *in vitro* release with a burst release of ~1,800 µg on day 1 which rapidly declined to ~380 µg on day 4 after which more stable release kinetics were observed with implants prepared with 35% PEG. Drug release was found to be 300-350 µg/day for over a period of 1 month and then reduced to ~130 µg/day after 3 months. Cumulative *in vivo* release was ~30% after 1 month which was ~1.8-2 fold higher than *in vitro* release suggesting a good correlation between simulated *in vitro* and *in vivo* conditions. Furthermore, only ~50% (19 mg out of 38.2 mg determined in initial implants) drug was found to be released from these implants over a period of 3 months showing their potential to deliver curcumin for extended periods of time ranging from 6 months to a year.

Table 3.1.

Cumulative *in vivo* curcumin release from implants containing 20% (w/w) drug load and 10% (w/w) F-68 for a period of 16 months.

		Cumulative Drug Release (mg)					Cumulative Drug Release (%)					Average Daily Release (µg)				
Weeks	**Days**	**Imp 1**	**Imp 2**	**Imp 3**	**Mean**	**SD**	**Imp 1**	**Imp 2**	**Imp 3**	**Mean**	**SD**	**Imp 1**	**Imp 2**	**Imp 3**	**Mean**	**SD**
1	7	5.78	4.69	3.53	4.66	1.12	15	12	9	12	3	825.02	669.72	504.06	666.27	160.51
2	14	6.74	8.00	7.28	7.34	0.63	18	21	19	19	2	295.91	476.61	374.31	382.28	90.61
3	21	10.26	8.75	9.18	9.40	0.78	27	23	24	25	2	417.19	202.06	262.89	294.05	110.90
5	35		12.37	12.07	12.22	0.21	0	32	32	21	19		211.95	190.69	201.32	15.03
8	56	15.03	15.90	15.04	15.32	0.50	39	42	39	40	1	134.01	175.21	134.49	147.90	23.65
18	126	20.61	17.40	18.81	18.94	1.61	54	46	49	50	4	75.47	29.69	49.82	51.66	22.95
36	252	23.67	24.62	23.84	24.05	0.51	62	65	63	63	1	37.59	45.12	38.89	40.53	4.02
42	294	24.87		25.58	25.22	0.50	65		67	66	1	19.55		36.47	28.01	11.96
70	490	28.67	29.25	28.63	28.85	0.35	75	77	75	76	1	17.57	20.55	17.41	18.51	1.77

Table 3.2

Cumulative *in vivo* curcumin release from implants containing 20% (w/w) drug load and 35% (w/w) PEG for a period of 6 months.

	Cumulative Drug Release (mg)					% Cumulative Drug Release					Average Daily Release (µg)				
Days	**Imp 1**	**Imp 2**	**Imp 3**	**Mean**	**SD**	**Imp 1**	**Imp 2**	**Imp 3**	**Mean**	**SD**	**Imp 1**	**Imp 2**	**Imp 3**	**Mean**	**SD**
1	2.23	1.44	1.75	1.8	0.4	5.8	3.8	4.6	4.7	1.0	2230.0	1440.0	1750.0	1806.7	398
4	2.65	2.49	3.51	2.9	0.5	6.9	6.5	9.2	7.5	1.4	282.2	230.7	568.7	360.6	182
12	5.84	6.05	7.80	6.6	1.1	15.3	15.8	20.4	17.1	2.8	368.1	393.4	612.3	457.9	134
25	10.91	10.75	11.00	10.9	0.1	28.5	28.1	28.7	28.4	0.3	331.3	319.3	338.7	329.8	10
90	18.84	19.39	18.91	19.0	0.3	49.2	50.6	49.4	49.7	0.8	124.1	132.6	125.1	127.3	5
138	23.04	24.73	23.03	23.6	1.0	60.2	64.6	60.1	61.6	2.6	82.4	117.0	82.2	93.9	20
184	24.53	25.37		25.0	0.6	64.1	66.2		65.1	1.5	58.3	67.0		62.7	6

Tissue Distribution of Curcumin

Tissue concentrations of curcumin delivered either by implants or by dietary routes were also measured in plasma, liver and brain. For these studies, female ACI rats were either implanted with two 2-cm implants (200 mg, 20% drug load) or were administered with diet containing curcumin (1,000 ppm). The animals were sacrificed after 1, 4, 12, 25 and 90 days and various tissues (plasma, liver and brain) were collected. Curcumin was extracted from the tissues using solvent extraction and analyzed using HPLC coupled with a fluorescence detector with an extraction efficiency of ~90% from plasma and ~70% from tissues (liver and brain). Fluorescence detector was used due to very high fluorescence of curcumin which not only increased the specificity of curcumin detection but also increased the sensitivity by at least 4-5 fold. The lower limit of detection in plasma was found to be 125 pg (340 pM) and limit of quantification was ~200 pg (540 pM) by this method.

When ~500 µl of plasma was extracted from each individual animal and analyzed by HPLC, the levels were found to be very low and were below the quantification limit of the method. Therefore, to increase the specificity and reliability of quantification, the extraction was performed with 1.5 ml plasma by pooling 250-350 µl of plasma from all the animals (4-6 in each group) (Figure 3.3). The plasma curcumin levels from the implants group were found to be 3.3 ng/ml (9 nM) on day 1 which decreased rapidly to 1.4 ng/ml (4 nM) on day 4 after which a slow and steady decrease in plasma concentration was observed reaching ~0.2 ng/ml (543 pM) after 3 months. Curcumin diet on the other hand, showed different kinetics in that curcumin was detected only on day 4 at a level of 0.3 ng/ml (815 pM) decreasing to 0.2 ng/ml after 12 days (543 pM) and was undetectable after 25, and 90 days. It was highly counter intuitive that instead of the fact that curcumin diet consumption was constant at all the time periods, curcumin was not

observed on day 1 and a decline in plasma curcumin concentrations was observed on day 4 suggesting time- and exposure-dependent absorption kinetics. Furthermore, curcumin due to its lipophilic nature exhibits biphasic elimination kinetics with rapid equilibration and distribution of plasma curcumin into various tissues (121). It is, therefore, also possible that due to high tissue distribution of curcumin, initially almost all curcumin equilibrates into tissues and was not observed in plasma on day 1. However, with time as the lipophilic tissues gets saturated, curcumin was observed in plasma after 4 days on dietary administration.

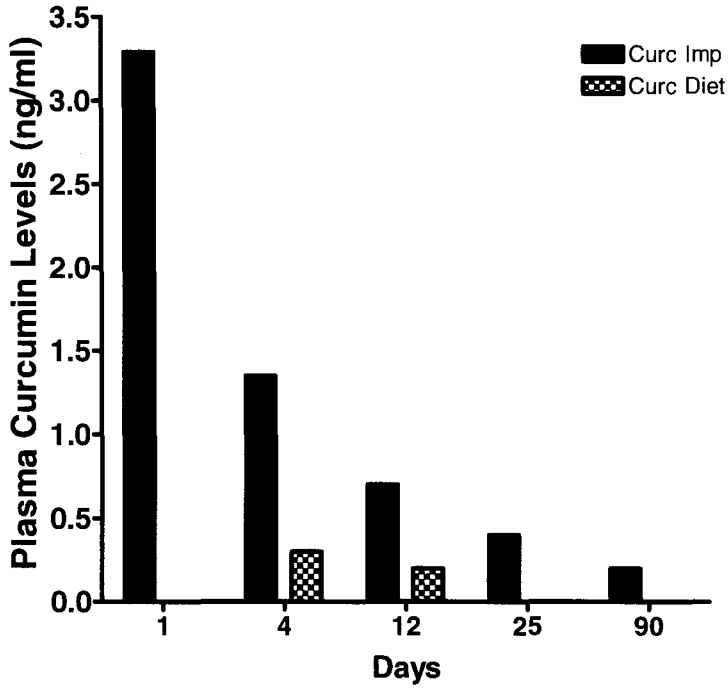


Figure 3.3. Curcumin levels in the plasma of ACI rats treated with curcumin implants (two 2-cm implants; 20 mg curcumin/cm) or curcumin diet (1,000 ppm). One and half ml plasma was extracted from each time point after pooling and analyzed by HPLC using fluorescence detector as described under methodology.

It is known that curcumin possess poor oral bioavailability owing to its low aqueous solubility (24) and rapid metabolism both in intestine as well as in liver (12, 122). Therefore, to further understand the kinetics of curcumin delivered *via* both the routes,

its concentration in liver was also measured (Figure 3.4). Curcumin delivered *via* both routes was found to be at similar levels in liver and was 25-30 ng on day 1. The levels increased slightly, though insignificantly on day 4 after which the levels declined to 10-15 ng after 12 days. This trend was found to exactly mimic the plasma concentrations suggestive of hepatic regulation of plasma curcumin levels at least *via* dietary route. Curcumin is known to induce the expression and activity of various enzymes like CYP1A1 (123), GSTM (124), CYP3A4 (125) and it appears that significant amounts of curcumin that reaches the liver tissue induce its own metabolism by increasing the expression/activity of these xenobiotic metabolizing enzymes. The levels, therefore, decreases significantly after 12 days and stayed almost constant afterwards. Furthermore, curcumin concentration was found to be much higher (10-20 fold) in liver as compared to plasma via both routes again suggestive of its high tissue distribution. High curcumin concentrations in hepatic tissues via dietary administration were expected as most of the orally administered curcumin gets absorbed into the liver where it undergoes rapid metabolism. However, similar behavior from the implant route was unexpected as it bypasses the hepatic first-pass metabolism and almost all of the administered curcumin directly reaches into the systemic circulation. Therefore, a much higher concentration was expected in plasma from implant route. It appears that such a pharmacokinetic behavior also originated from curcumin's high lipophilicity resulting in high tissue distribution as compared to plasma. Moreover, since physiologically liver is a highly perfused organ and gets around 23% of total cardiac output, it is highly probable that most of the curcumin that reaches into the systemic circulation might get rapidly equilibrated and entrapped into the liver tissue. An almost constant liver concentration of curcumin from 12 to 90 days also suggests tissue binding of this compound.

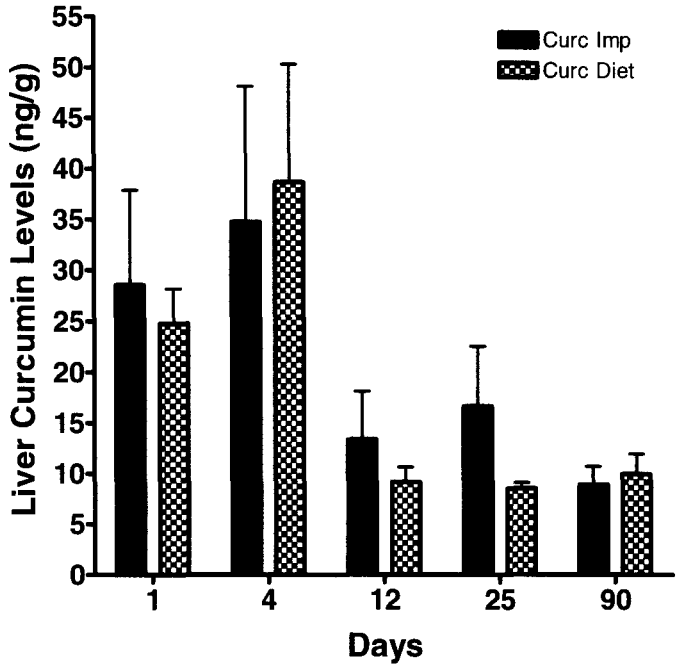


Figure 3.4. Liver curcumin levels in ACI rats treated with curcumin implants (two 2-cm implants, 20 mg/cm implant) or curcumin diet (1,000 ppm). Tissue (~500 mg) from individual animal (n =4-6) was extracted and analyzed by HPLC coupled with a fluorescence detector as described in methodology.

To support these observations, we further analyzed curcumin concentration in the whole brain tissue as it is also one of the highly perfused organs. Brain was also selected because of curcumin's known potent activities against Alzheimer's disease (126) and against brain gliomas (127) where continuous localized/systemic delivery of this compound could make a significant improvement in the life of such patients. Analysis of brain tissue showed almost constant levels of curcumin (2-3 ng/g) from dietary administration at all the time points (Figure 3.5.). Although, curcumin was not found in plasma on day 1 in the dietary curcumin group, it indeed was present in brain. This observation further supports the fact that initially all the curcumin that escaped from liver and reached the systemic circulation partitioned into highly lipophilic sites like liver and other tissues. However, as equilibrium is established between plasma and other organs like brain, curcumin started to accumulate in plasma and could be detected after 4 days

of oral administration. Furthermore, constant curcumin levels in brain detected even after 90 days again suggests increased metabolism of curcumin in liver tissues that led to decreased curcumin concentration in liver at 12 days.

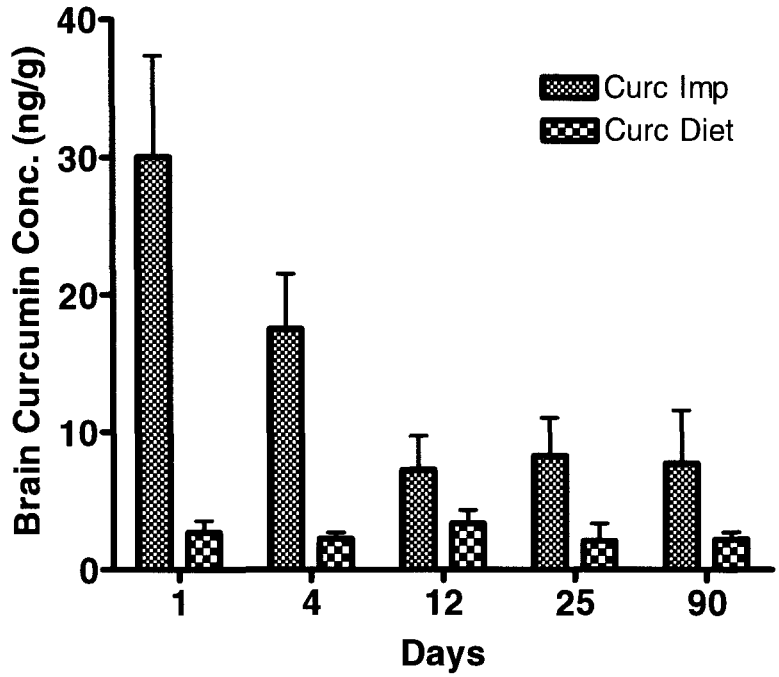


Figure 3.5. Brain curcumin levels in ACI rats treated with curcumin implants (two 2-cm implants, 20 mg/cm) or dietary curcumin (1,000 ppm). Tissue (~500 mg) from individual animals was extracted and analyzed by HPLC coupled with a fluorescence detector. Data represent average of 4-6 animals, except for day 1 where data represents average of two animals since curcumin was below the detection limit in the remaining animals.

On the other hand, curcumin delivery to the brain via implant route showed kinetics similar to plasma. Since, drug concentration that reaches a tissue is determined by rate and extent of its perfusion by systemic circulation, brain showed rather high curcumin levels (30±7.3 ng/g tissue), almost equal to liver concentration on day 1 of implantation. This concentration dropped slowly to around 7.26±2.45 ng/g tissue after 12 days and stayed almost at the same levels even after 90 days which was slightly less than the liver concentration from 12 to 90 days (a constant drug concentration period). It is known

that physiologically, liver gets ~23% of cardiac output and brain gets slightly less than liver (~18%). This fact again shows that tissue distribution from implant route was indeed perfusion rate limited and is dictated by cardiac output to that particular organ. It may be emphasized that curcumin concentration in the brain tissue was ~3.5-fold higher by the implant route in which case total curcumin administered was nearly 25 fold lower compared to oral administration over a period of 3 months.

Analysis of curcumin in different tissues showed that plasma curcumin concentration *via* dietary administration is regulated by liver. At high liver concentration, a fraction of dietary curcumin escapes hepatic first pass metabolism into the systemic circulation and gets distributed to other organs. Tissue concentration is then determined by extent of blood supply to the respective organ. However, this is not the case with the implant route where plasma is the first site of curcumin absorption from where it gets simultaneously distributed to all the other tissues. Tissue concentrations are, therefore, determined only by the extent and rate of blood that supplies the organ. In view of these facts, it is possible that to administer curcumin to other organs like prostate or pancreas which are not richly supplied with blood, dietary administration might not be an appropriate route. In such cases, direct systemic administration either via parenteral routes or via implant route would be a better alternative that can be further evaluated for various preclinical and clinical studies.

Modulation of Phase I and Phase II Enzymes

CYP1A1 and GSTM (μ) Expression Analysis in Hepatic Microsomes

Various *in vitro* and cell culture studies showed that curcumin's effective concentration is 5-20 μM depending upon the cell line used (128). Since the plasma and liver

concentrations observed in our *in vivo* study (70-90 nM) were well below these, we also determined if these concentrations modulated hepatic cytochromes. Studies have shown that curcumin is a natural agonist of aryl hydrocarbon receptor (AhR)/pregnane xenobiotic receptor (PXR) pathway and induces the expression of phase I enzymes like CYP1A1 (123) and CYP3A4 (125). Furthermore, it also interacts with the keap1 protein to lower its affinity for nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein followed by Nrf2 transloaction into the nucleus to induce phase II enzymes like glutathione-S-transferase(μ) (GSTM) (124). Therefore, we isolated hepatic microsomes and analyzed them for their CYP1A1, CYP3A4 and GSTM levels by western blotting and activity of CYP3A4 (a major drug metabolizing enzyme) after 1, 4, 12, 25 and 90 days of curcumin treatment by both dietary and implant routes.

As is evident from the Figure 3.6, curcumin implants were found to induce expression of CYP1A1 by ~2 fold after 4, 12, 25 and 90 days of treatment. Although, a 2 fold increase in CYP1A1 was observed on day 1, yet the effect was masked by PEG as a similar effect was also observed in blank implants at this time point and this effect from the blank implants was absent at all other time points. This effect from blank implants was presumably due to release of significant amounts of PEG on day 1 which decreased significantly on day 4 (PEG release was measured by subtracting the amount of released curcumin and weight of dried implant recovered from animals from initial weight of the implants) (129). Curcumin diet, on the other hand, increased CYP1A1 levels only slightly on day 1 (1.43 fold) which returned to basal levels after 4 days and in-fact the levels were down-regulated after 25 and 90 days of treatment. Since, the hepatic curcumin levels were similar by both the routes tested; such route-dependent differences in induction of CYP1A1 expression were counter intuitive. It is known that various chemopreventives like indole-3-carbinol (I3C) are most effective in inducing CYP1A1

when given orally as compared to when given systemically (130). It has been found that polymerization products of indole-3-carbonol like diindolylmethane formed under stomach acidic conditions are more effective AhR ligands as compared to the parent compound, thus resulting in higher efficacy of indole-3-carbinol (130). Curcumin is also known to degrade under slightly basic conditions of intestine forming various active degradation products like ferulic acid and vanillin (92). It is possible that one or more of these metabolites might have AhR antagonistic activity resulting in blunting of curcumin's CYP1A1 upregulating activity at initial time points and eventual downregulation after 25 and 90 days of treatment. GSTM, on the other hand was not modulated *via* either of the routes (data not shown).

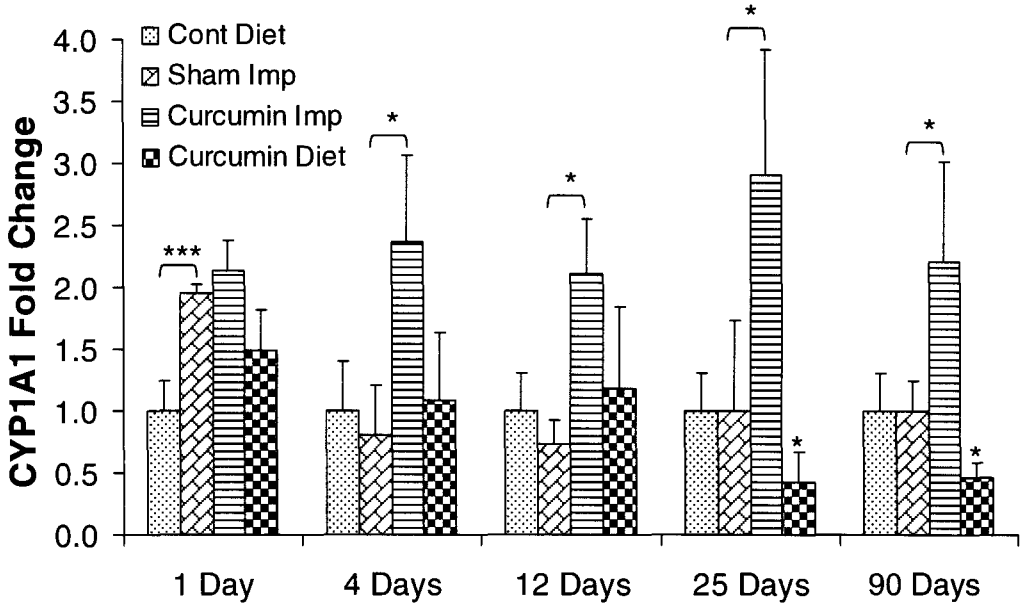


Figure 3.6. Effect of curcumin administered by polymeric implants (two 2-cm implants, 20 mg/cm) or diet (1,000 ppm) on CYP1A1 protein expression in hepatic microsomes of ACI rats. Blank implants and curcumin diet groups were compared with untreated controls and curcumin implants were compared with blank implants for all statistical purposes at a significance level of p value <0.05 (n=3-4).

CYP3A4 Activity and Expression Analysis in Hepatic Microsomes

Effect of curcumin on CYP3A4 activity was also measured as it is the major drug metabolizing enzyme known to get induced by curcumin (Figure 3.7) (125). CYP3A4 activity was found to be increased significantly by curcumin diet after 4 days but came to basal levels after 12 days of treatment. Curcumin implants, on the other hand, were found to increase the activity only slightly on both day 1 and day 4 of treatment. However this increase became significant after 12 days of treatment and remained upregulated up to 3 months. This increase was not found to blunt with time and increased activity was found up to 3 months. It appears that continuous delivery of curcumin directly into the systemic circulation by the implant route was more effective in increasing and maintaining the higher CYP3A4 activity as compared to dietary route.

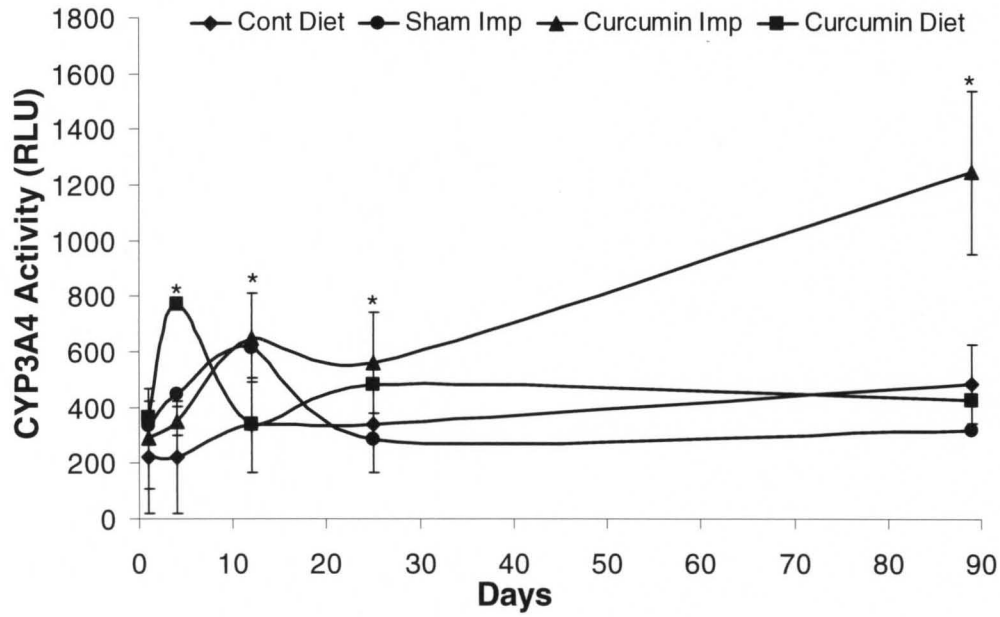


Figure 3.7. Effect of curcumin administered by polymeric implants (two 2-cm implants, 20 mg/cm) or diet curcumin (1,000 ppm) on CYP3A4 activity in hepatic microsomes of ACI rats. Blank implants and curcumin diet groups were compared with untreated controls and curcumin implants were compared with blank implants for all statistical purposes at a significance level of p value <0.05 (n=3-4).

Conclusions

These studies showed that polymeric implants on subcutaneous implantation can provide a controlled release of curcumin at the local site from which it gets absorbed into the systemic circulation and gets distributed to all the tissues simultaneously. These implants were found to provide higher curcumin concentrations in plasma, brain and to some extent in liver over a period of 3 months as compared to dietary administration. Furthermore, curcumin delivered directly into the systemic circulation was found to be more efficacious in inducing CYP1A1 and CYP3A4 enzymes required for its chemopreventive activity against various environmental carcinogens. Presence of significantly higher concentrations of curcumin in tissues like brain shows potential of this delivery system for patients suffering with Alzheimer's disease and/or brain gliomas where curcumin has shown significant potential in various *in situ* and cell culture studies but does not reach in sufficient amounts by dietary route.

CHAPTER IV

CURCUMIN IMPLANTS FOR CONTINUOUS SYSTEMIC DELIVERY: SAFETY AND BIOCOMPATIBILITY

Rationale

Biomedical implants, once implanted subcutaneously, come in contact with blood and tissues often initiating immunological reactions by activating cellular and humoral immune responses. This is a surface phenomenon, characterized by various cell polymer, protein-polymer and cell-cell interactions. Although, poly(ε-caprolactone) (PCL) is an FDA–approved, biocompatible polymer for use in humans (104) and its contraceptive implants are in phase II clinical trials in China (131), various implant attributes like implant shape, size, surface roughness, porosity, composition and nature of degradation all contribute towards the intensity and duration of tissue reactions (132). Therefore, determination of biocompatibility and immunological safety of these PCL implants containing 35% polyethylene glycol, molecular weight 8,000 (PEG) is another criterion for selection of suitable implant candidates for further evaluation under preclinical and clinical studies. Moreover, although curcumin was not found to induce any observable toxicity when given orally to patients and was well tolerated in doses 8-12 g (133), its toxic effects on direct systemic administration of high doses are unknown. However, recently Dandekar et al. (134), showed safety of oral curcumin NPs made by using Eudragit® S100 but the studies were done by oral administration and does not provide insights about the systemic administration of curcumin. Since, polymeric

curcumin implants release ~300-500 µg/implant/day curcumin directly into the systemic circulation at least initially for 2-3 weeks (135), we wanted to determine if subcutaneous administration of such high quantities of curcumin exert any biochemical alterations in body by altering liver and kidney functions. Therefore, this study was designed to examine the systemic and local effects of the polymeric implants formulated with and without curcumin. Blood clinical chemistry, various hematological parameters and systemic biochemical parameters of liver and kidney function were monitored to assess the safety, tolerability and biocompatibility of implants. Local site of implantation was also evaluated histologically to determine the degree and extent of inflammation induced by these implants as well as by systemic delivery of high doses of curcumin to determine their biocompatibility and acceptability by the body.

Materials and Methods

Materials

Medical grade PCL of 121,000 molecular weight (PCL) was purchased from SurModics Pharmaceuticals (Birmingham, AL). Dichloromethane (DCM) was purchased from Sigma-Aldrich (St. Louis, MO), polyethylene glycol of 8000 molecular weight (PEG) from Fisher Scientific (Fair Lawn, New Jersey), and ethanol from Pharmco-AAPER (Louisville, KY). Silastic tubing (3.4 mm internal diameter) was purchased from Allied Biomedical (Ventura, CA). Curcumin (C-3 complex) extracted under GMP conditions (good manufacturing practices) was a generous gift from Sabinsa Corporation (East Windsor, NJ). All the materials were used as received without any further analysis.

Methods

Preparation of Curcumin Implants

Curcumin implants were prepared by using a combination of solvent evaporation and melt-extrusion technique as described in chapter 2. Briefly, curcumin was dissolved in ethanol and polymers (PCL with PEG in a ratio of 65:35) were dissolved in DCM. Two solutions were then mixed together and solvents were evaporated at 70 °C to prepare a homogenous molecular dispersion of drug in polymeric matrix followed by overnight drying under vacuum at 65 °C. The dried molten polymer was extruded through silastic tubing attached to a syringe. After few min, the cylindrical implants were removed from the tubing and excised into desired lengths (2 cm, 200 mg with 20 mg curcumin/cm) and stored at -20 °C under argon until used.

In Vivo Studies

In vivo release, biocompatibility and toxicity studies were performed in female ACI (Augustus Copenhagen Irish) rats in accordance to an approved protocol from the Institutional Animal Care and Use Committee (IACUC). Animals were provided with purified diet; AIN-93M and water *ad libitum* until termination of the study. After acclimation of animals to vivarium conditions, either two 2-cm implants with or without (sham implants) 20% (w/w) curcumin (20 mg/cm) were subcutaneously grafted at the back of the rats as described previously (119) or were provided with curcumin (1,000 ppm) mixed with diet. Animals were euthanized at different intervals by asphyxiation to collect blood (by cardiac puncture), liver, local tissues and implants from the implantation site. Implants recovered from the animals were dried overnight under vacuum, weighed and stored under argon at -80 °C until analysis of the residual curcumin content. A small portion of liver from the biggest lobe and a portion of tissue from the site of implantation were fixed in formalin and the remaining tissues were snap frozen in liquid nitrogen and stored at -80 °C for future use. Whole blood was used to measure the hematological parameters by Cell-Dyn 3500 hematology analyzer (Abbott laboratories, Santa Clara, CA) which employs Multi-Angle Polarized Scatter Separation technique to provide primary white blood cell count, reticulocyte analysis and all differential counts. Serum was used to measure various biochemical parameters of liver and kidney function by using an automated AU640® Chemistry Analyzer (Beckman Coulter, Inc. Brea, CA) that uses an ion selective electrode to measure various electrolytes and in built spectrophotometric techniques for all other assays. These assays were done in a local diagnostic lab maintained and run by Antech Diagnostics.

Residual Drug Determination

Implants (n=3) collected after 1, 4, 12, 25 and 90 days at the time of euthanasia were analyzed to determine the residual drug content, as described in chapter 3. Briefly, implants recovered from animals were dried over night under vacuum, were weighed and dissolved in 5 ml DCM. To this solution 5 ml ethanol was added to completely dissolve curcumin. The solution was then diluted suitably (1:10) with ethanol containing 20% DCM followed by in ethanol (1 in 40). Drug concentration was measured by UV spectrophotometer at 430 nm to calculate the amount of curcumin released *in vivo* using following equation:

$$\text{Cumulative curcumin release} = \frac{\text{Initial amount - Residual amount}}{\text{Days}}$$

Histology studies

Tissues, collected in 10% buffered formalin, were transferred to 70% ethanol and processed at the University's core histology facility. Paraffin sections of 5-µm thickness were cut and stained with hematoxylin and eosin. Slides were blinded and observed under light microscope by an experienced cytopathologist.

Statistics

Two way Anova with Bonferroni post-test was used to calculate statistically significant differences between the various interventions and a p value of <0.05 was considered as significant.

Results and Discussion

Acute and chronic local/systemic toxicity was studied by subcutaneously implanting two, cylindrical PCL-PEG implants (200 mg each) with or without curcumin for a period of 3 months. Systemic toxicological effects were determined by measuring various hematological parameters, serum biochemical parameters of liver & kidney function and the local effects of implantation were evaluated by histological examination of the implant site. After specific time intervals (1, 4, 12, 25 and 90 days), 4-5 animals from each group were euthanized, blood/serum was collected by cardiac puncture and analyzed for various biochemical parameters to determine the effects of polymeric implant formulation (sham implants) and of continuous administration of curcumin delivered directly into the systemic circulation by these implants. Gross examination of liver, lung, kidney, brain and pituitary was also carried out at the time of necropsy and were weighed to determine any differences as compared to untreated animals.

All rats in the study were found to be healthy and active without any evident pathological manifestations irrespective of the kind of treatment given during the whole period of study. However, pre-necroptic palpation of animals showed slightly thickened areas over the implant sites without any apparent effect on hair growth and texture. No statistically significant differences were found either in diet intake (Figure 4.1) or in weights of different organs (liver, lung, kidney, brain, pituitary and mammary tissue) over a period of 3 months. However, a reduction in body weight gain was observed in animals treated with curcumin either *via* implants or *via* diet (Figure 4.2). Curcumin has been shown to possess hypoglycemic (136) and hypocholesterolemic activities (137) and it appears that curcumin when delivered *via* either route was able to exert these activities.

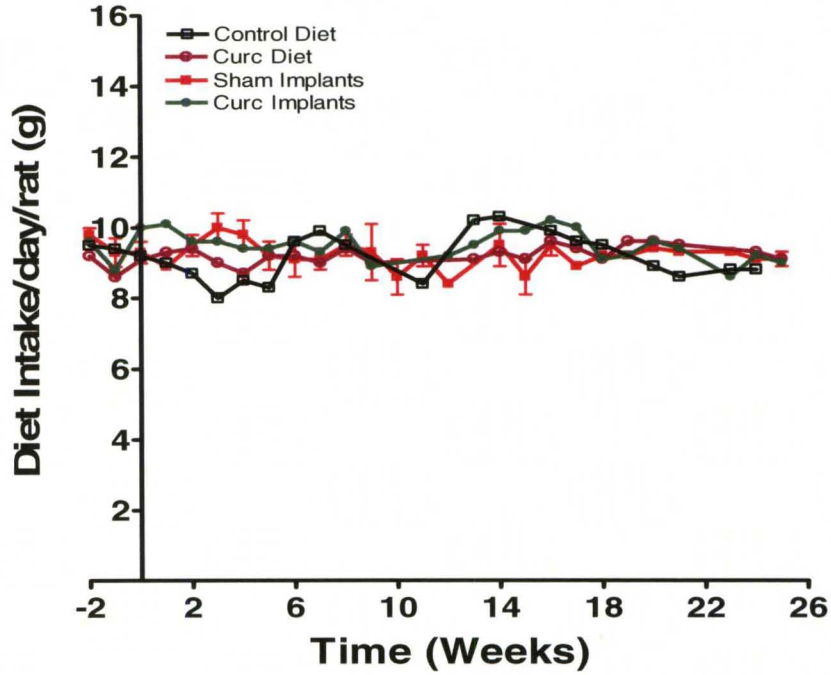


Figure 4.1. Diet intake of animals treated with either curcumin diet (1,000 ppm), sham implants or with curcumin implants as compared to animals given control diet AIN-93M.

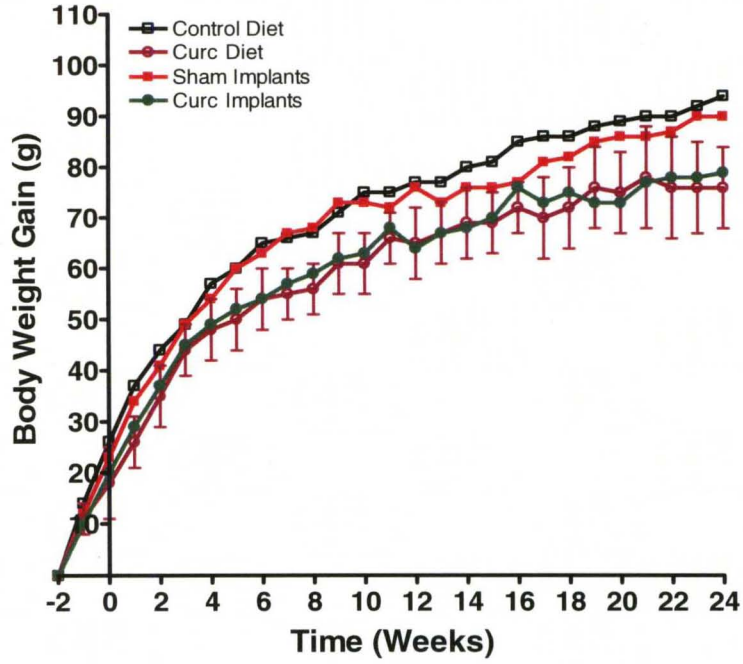


Figure 4.2. Body weight gain of animals treated with either curcumin diet (1,000 ppm), sham implants or with curcumin implants as compared to animals given control diet; AIN-93M.

Cumulative Drug Release

Since the polymeric implant drug delivery system enabled us to continuously deliver high quantities of curcumin directly into the systemic circulation, we determined the amount of curcumin released under *in vivo* conditions by measuring the residual amount of drug in implants recovered from animals. It was found that a total of 3.6 ± 0.8 mg of curcumin was released from both the implants combined (4.5% of total drug load from each implant) on day 1 of implantation and increased to 5.8 ± 1.1, 13.1 ± 2.1, 21.8 ± 0.3, 38.1 ± 0.6, 47.2 ± 1.6 mg after 4, 12, 25 and 90 days respectively (Figure 4.3).

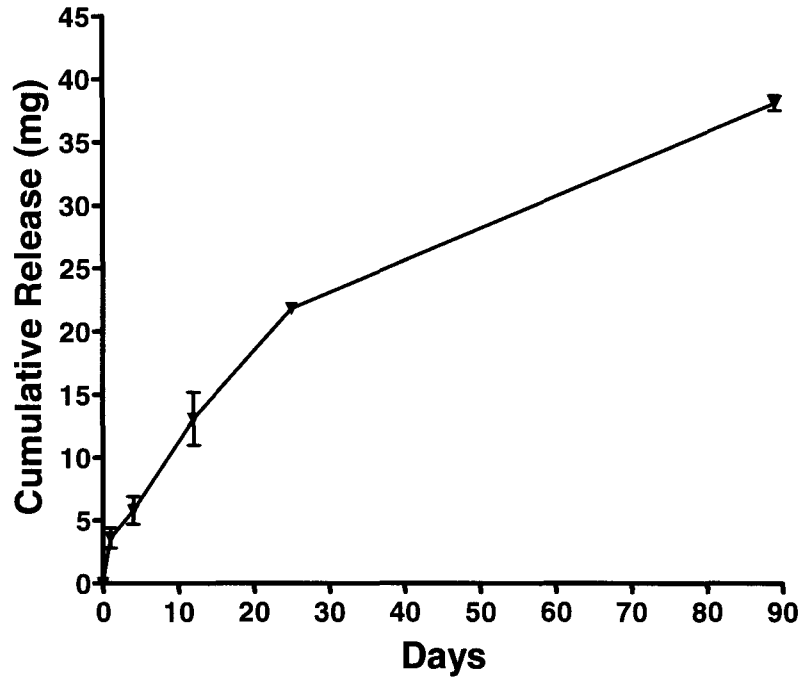


Figure 4.3. Cumulative curcumin release from two 2-cm implants (200 mg with 40 mg drug in each) grafted in female ACI rats. Data represent twice the average release from implants recovered from 3-4 animals (±SD).

Since curcumin released from implants at the site of implantation gets absorbed into the systemic circulation, it is clear that high amounts of curcumin entered into blood and got distributed to various tissues. Since, liver and kidney are the highly perfused organs specific for elimination of xenobiotic compounds and are highly sensitive to xenobiotic

insults; we measured various (enzyme and electrolyte) indicators of liver and kidney function at different time intervals after treatment with curcumin implants and compared them with curcumin diet (1,000 ppm).

Effect on Liver Enzymes

AST, ALT, ALP, GGT, amylase and lipase levels were measured in serum of animals treated either with two 2-cm PCL implants (200 mg) containing PEG (35% w/w) alone (sham implants) or curcumin (20% load) with PEG (35% w/w) or curcumin diet (1,000 ppm) after 1, 4, 12, 25 and 90 days and were compared with untreated animals (Figure 4.4). Sham implants were found to decrease serum amylase levels after 1 day of implantation; however, this decrease was transient and returned back to normal levels after 4 days. Since sham implants contained 35% PEG which is a highly water soluble polymer, it appears that this transient increase in lipase levels might be due to release of high amounts of PEG into the systemic circulation on day 1 of implantation. This effect was neither found with curcumin delivered *via* implants suggestive of curcumin's hepatoprotective activity (138, 139) nor by dietary curcumin. Furthermore, no statistically significant effect was found on any of the other serum enzymes analyzed, with any of the treatments.

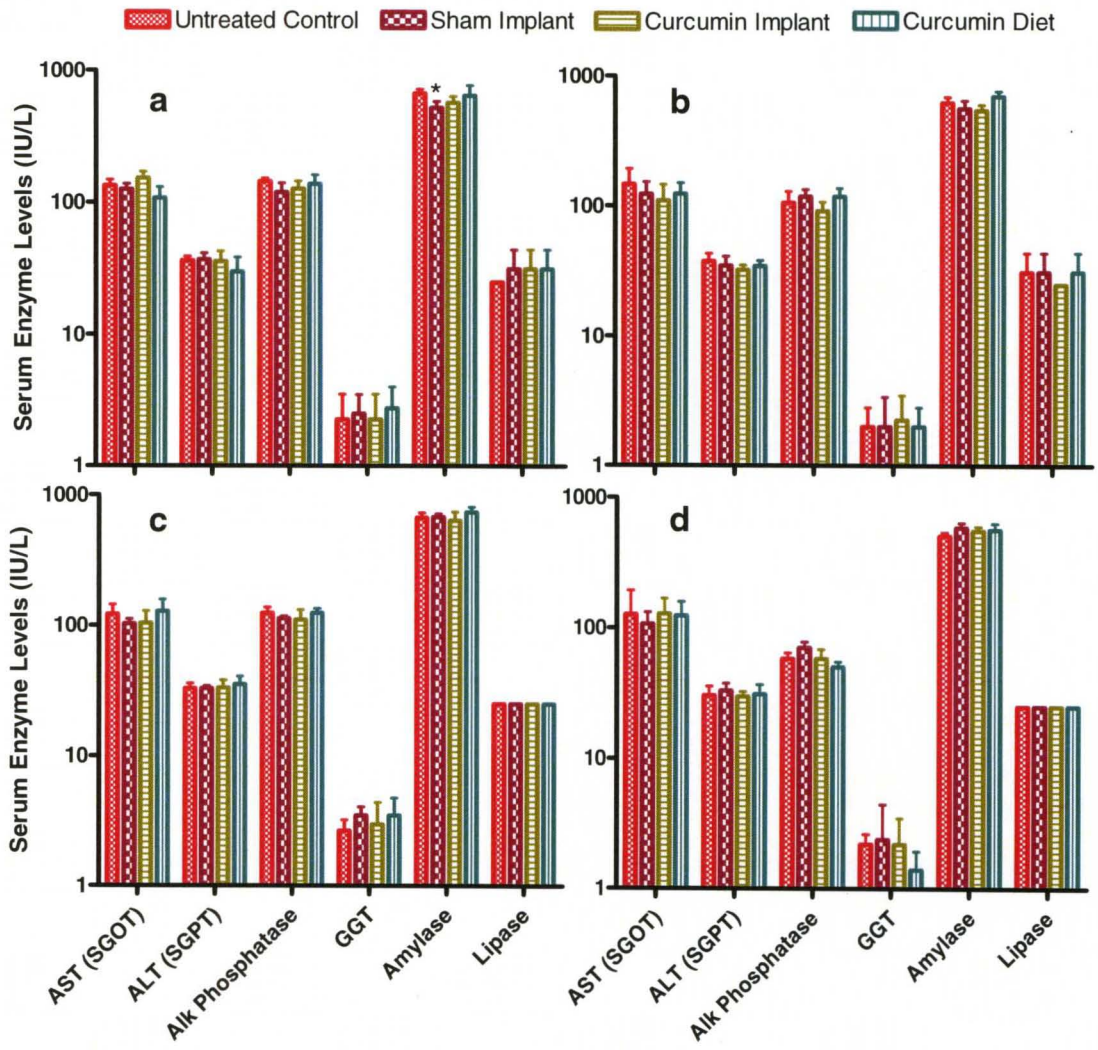


Figure 4.4. Various enzymes measured in serum of female ACI rats after subcutaneous implantation of two 2-cm polymer alone blank implants (sham implants containing PCL:PEG, 65:35), implants containing curcumin (40 mg each) and curcumin given in diet (1,000 ppm) after (a) 1 day, (b) 4 days, (c) 12 days and (d) 3 months; samples from 25 days were not analyzed.

Serum from animals was also analyzed for plasma proteins (albumin and globulin) as well as for effects on glucose, cholesterol and triglycerides (Figure 4.5.). Serum albumin levels were found to be slightly higher for sham implants and for curcumin diet after 12 days of treatment. However, this increase was not observed either at 4 or 25 days treatment or with curcumin implants. Furthermore, there was only 8.6% increase in

albumin levels and was well within the normal physiological range to indicate any major pathological conditions.

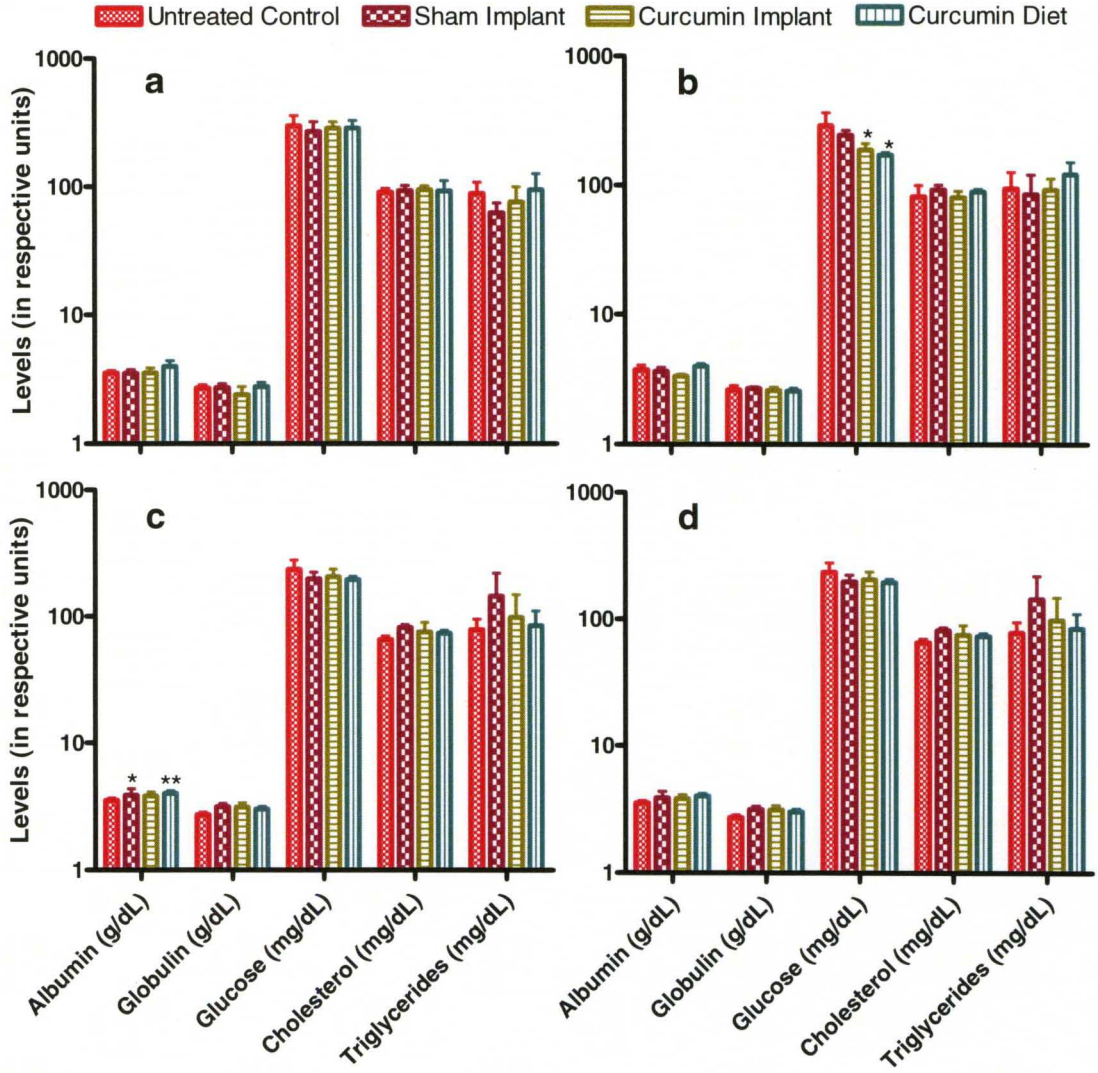


Figure 4.5. Plasma Proteins (albumin and globulin), glucose, cholesterol and triglycerides measured in serum of female ACI rats after subcutaneous implantation of two 2-cm polymer alone blank implants (sham implants containing PCL:PEG, 65:35), implants containing curcumin (40 mg each) and curcumin given in diet (1,000 ppm) after (a) 1 day, (b) 4 days, (c) 12 days and (d) 3 months; samples from 25 days treatment were not analyzed.

The most apparent effect of curcumin intervention was found to be on serum glucose levels. Curcumin delivered either *via* implants or in diet significantly reduced serum

glucose levels (188 ± 22 mg/dL and 172 ± 9 mg/dL, respectiverly) at 4 days of treatment compared to sham implant treatment (245 ± 22 mg/dL) or to untreated animals (289 ± 76 mg/dL) consistent with curcumin's anti-diabetic activity (136). Analysis of liver curcumin concentration delivered by both routes showed that curcumin levels increased slightly from 1 to 4 days (78 nM to 95 nM; assuming density of liver to be equal to 1), although not significantly, after which it decreased drastically at 12 days of treatment (35 nM) and was almost constant for up to 90 days. Since this effect was not observed either at 1 day or after 12 days of treatment, it appears that curcumin concentrations were high enough at 4 days to exert anti-diabetic effects and were low both at 1 day and 12 days of treatment. Furthermore, although TG levels were unchanged at all the time points shown, we have seen significant reduction of TG levels by curcumin both *via* implants (93 ± 22 mg/dL) and *via* diet (80 ± 27 mg/dL) after 6 months of treatment as compared to with sham treatment (166 ± 54 mg/dL) or with untreated control animals (143 ± 45 mg/dL). Both these observations suggest the usefulness of curcumin as an effective anti-diabetic as well as a hypocholesterolemic agent consistent with previous reports (137).

Effects on Serum Electrolytes/Kidney Function

Kidney function was analyzed by measuring blood urea nitrogen (BUN), creatinine and various electrolytes in serum (Figure 4.6). A transient increase in Mg^{2+} by curcumin diet and in K^{+} by sham implants was observed after 1 day of treatment and were normal at all the other time points. Both these effects were not observed with curcumin implants.

A more consistent alteration in phosphorus levels was also observed at all the time points studied. Phosphorus in serum of all the treatment groups; sham implants, curcumin implants and curcumin diet was found to be significantly elevated at all the time points. It is unclear at this point whether this effect is due to disturbances in

phosphorus absorption, storage or excretion and requires further investigation to determine the underlying cause(s).

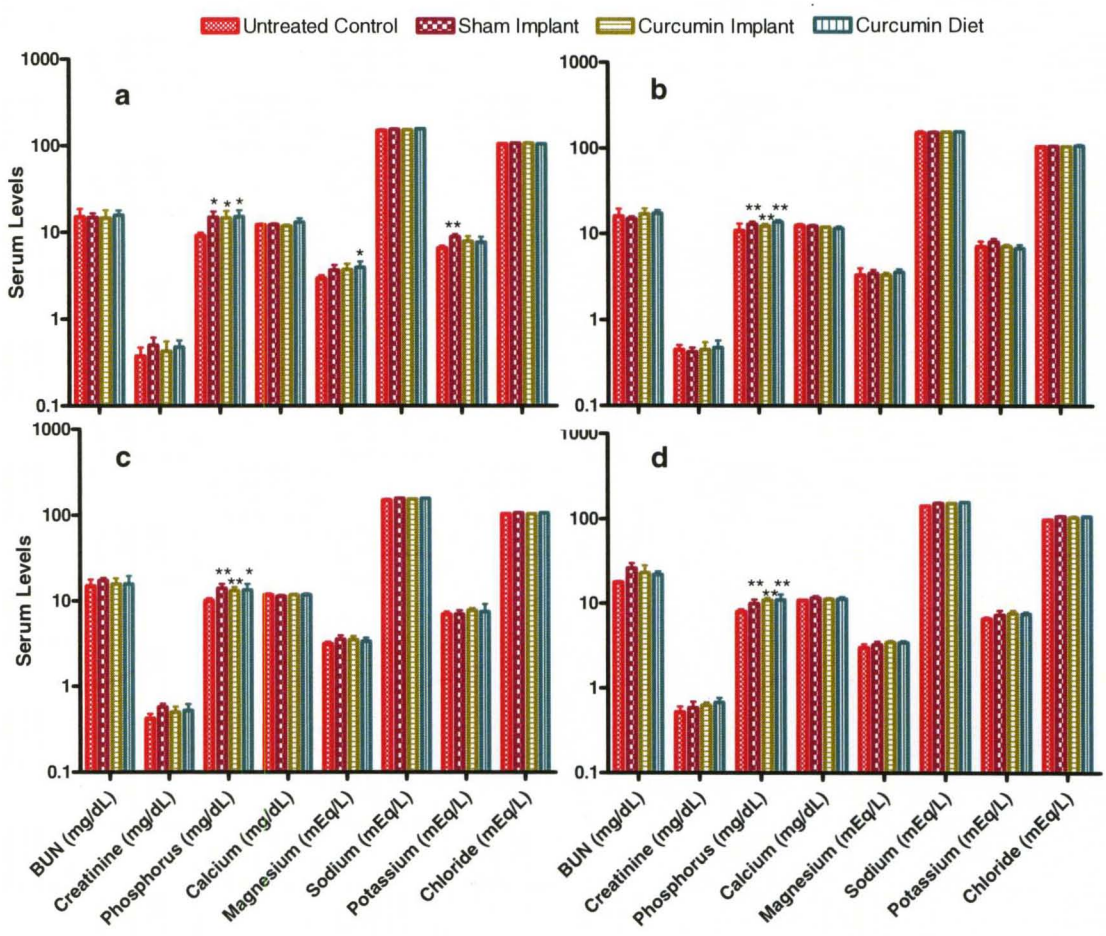


Figure 4.6. Various metabolites and electrolyte indicators of kidney function measured in serum of female ACI rats after subcutaneous implantation of two 2-cm polymer alone blank implants (sham implants containing PCL:PEG, 65:35), implants containing curcumin (40 mg each) and curcumin given in diet (1,000 ppm) after (a) 1 day, (b) 4 days, (c) 12 days and (d) 3 Months; samples from 25 day treatment were not analyzed.

Effect on Hematological Parameters

Whole blood from untreated and treated animals was also analyzed for various hematological parameters like white blood cells (WBCs), red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC)

and platelet count (Figure 4.7). No significant changes were observed in any of these parameters, except a slight decrease in platelet count and WBC count with curcumin diet at 4 days and 12 days time points, respectively. However, these effects were not observed either with sham or with curcumin implants at any of these time points. Moreover, platelet and WBC counts were found to be well within the baseline range of untreated animals over 90 days time period suggesting absence of any significant toxicity of PCL implants and hence of curcumin delivered *via* these implants.

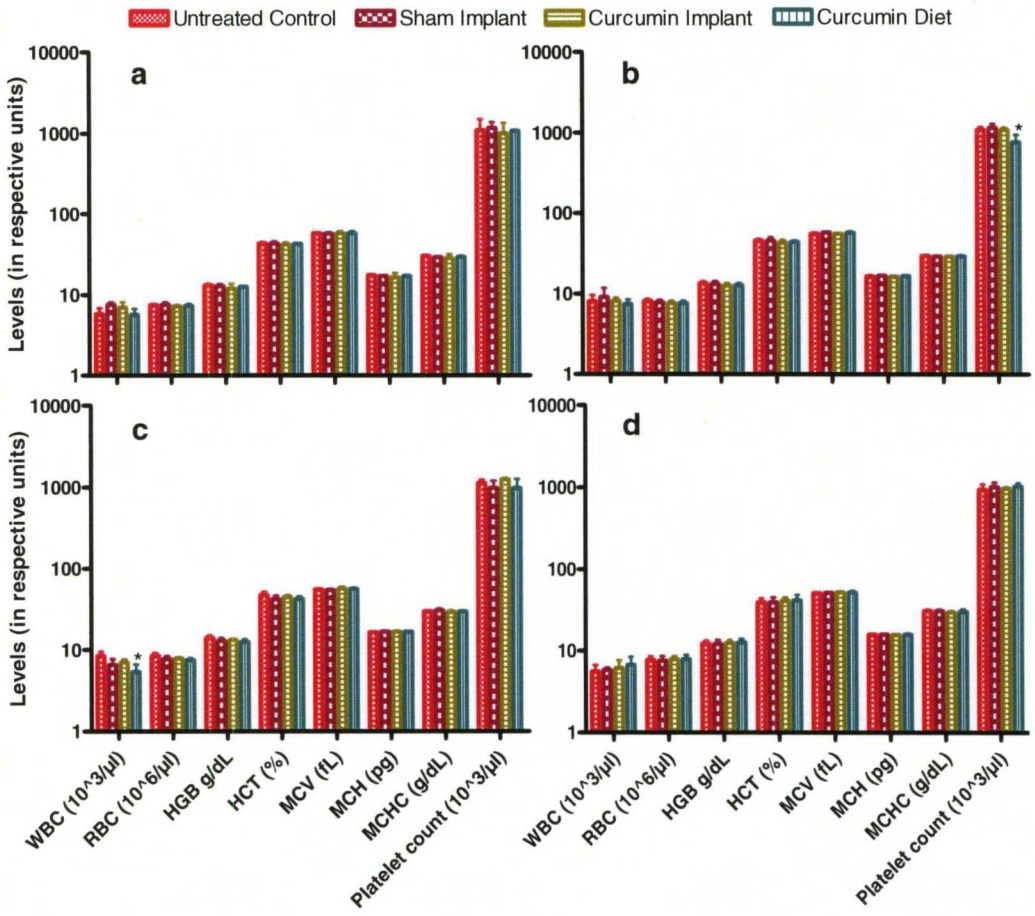


Figure 4.7. Various hematological parameters measured in blood of female ACI rats after subcutaneous implantation of two 2-cm polymer alone blank implants (sham implants containing PCL:PEG, 65:35), implants containing curcumin (40 mg each) and curcumin given in diet (1,000 ppm) after (a) 1 day, (b) 4 days, (c) 12 days and (d) 3 months.

Furthermore neutrophils, lymphocytes, monocytes, eosinophils and basophils were also analyzed in the whole blood. Although, no significant alterations were observed in monocytes, eosinophils and basophils, significant effects were observed in neutrophil and lymphocyte levels both with sham and curcumin implants at certain time points as compared to untreated animals (Figure 4.8). Therefore, to obtain a more realistic picture of fluctuations of these parameters in blood, both neutrophils and lymphocytes were analyzed by logistic transformation (Figure 4.9) as was suggested by Laurencin et al. (140). Logistic transformation was done to approximate the error distribution of measured values with the Gaussian reference distribution, using the following relation:

$$\text{Neutrophils} = \ln[\%\ \text{Neutrophils}/1-\%\text{Neutrophils}],$$

and

$$\text{Lymphocytes} = \ln[\%\ \text{Lymphocytes}/1-\%\text{Lymphocytes}]$$

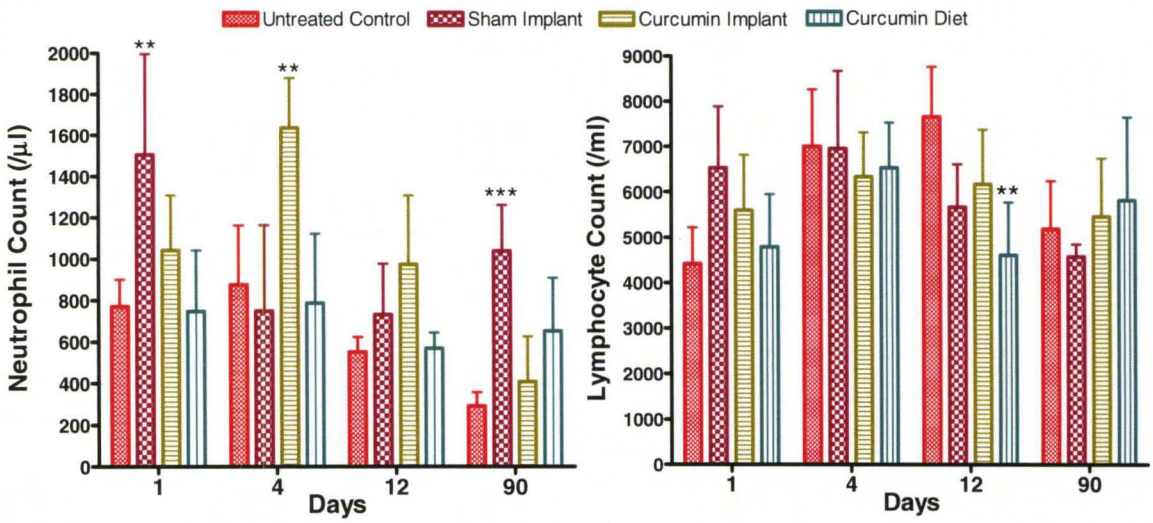


Figure 4.8. Neutrophil and lymphocyte counts (/µl) measured after 1, 4, 12 and 90 days in blood of (a) untreated female ACI rats or (b) after subcutaneous implantation of two 2-cm polymer alone blank implants (sham implants containing PCL:PEG, 65:35), (c) implants containing curcumin (20%, w/w) and (d) curcumin given in diet (1,000 ppm). Two-way ANOVA with Bonferroni post-test ($P<0.5$) was used to calculate statistically significant differences between the various interventions.

It is evident from Figure 4.9(a) that even the untreated animals exhibited significant fluctuations in the baseline levels of both neutrophils and lymphocytes and their levels in all the treatment groups were in baseline range of age-matched rats. However, since both neutrophils and lymphocytes are markers of acute and chronic inflammation, mild inflammatory reaction to these implantable foreign bodies at least at the local site is expected and is unavoidable as shown by Laurencin et al (140).

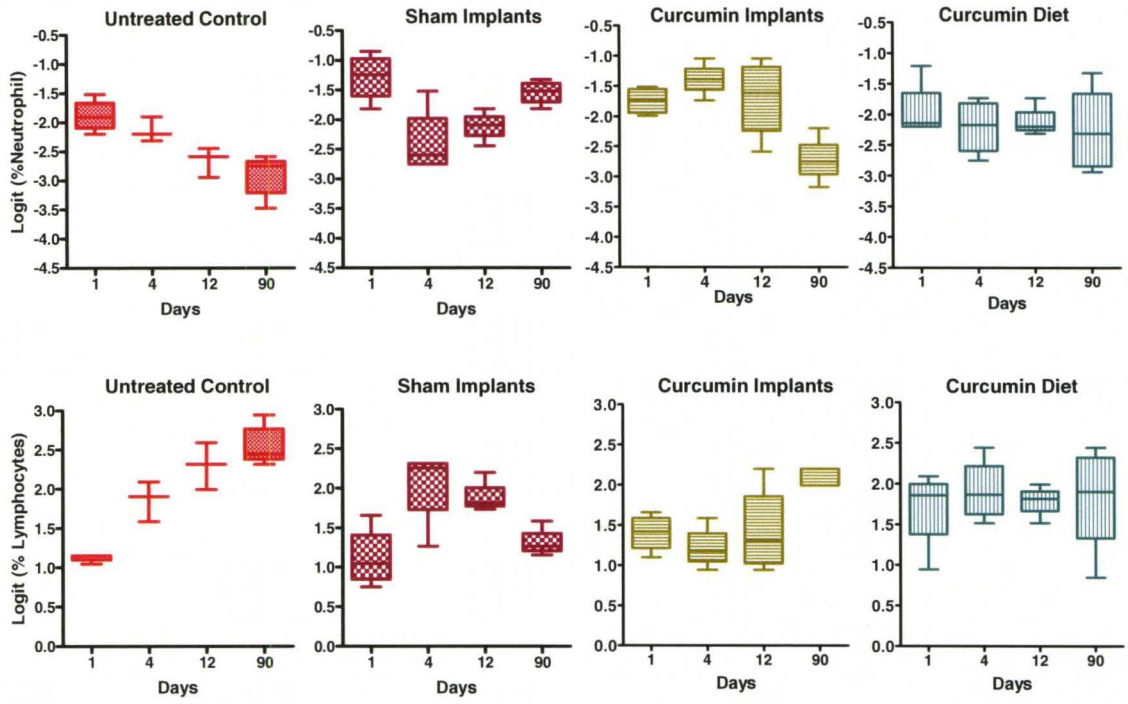


Figure 4.9. Neutrophil and lymphocyte counts expressed on a logistic scale, measured after 1, 4, 12 and 90 days in blood of (a) untreated female ACI rats or (b) after subcutaneous implantation of two 2-cm polymer alone blank implants (sham implants containing PCL:PEG, 65:35), (c) implants containing curcumin (20%, w/w) and (d) curcumin given in diet (1,000 ppm).

Evaluation of Local Implantation Sites

To further evaluate the effects of subcutaneous implants on the physical appearance or motility of animals and/or at the local site, detailed pre-necroptic and histological examination of the animals was carried out. All the animals were found to be physically

healthy and active without any visual evidence of any kind of polymeric material-associated toxicity even after 3 months of implantation of two 2-cm implants both with and without curcumin. Grossly, local implantation sites grafted with either sham implants (polymers alone) or curcumin implants appeared similar to the subcutaneous sites of untreated animals with only slight but insignificant thickened areas at the implantation sites. Normal hair growth without any redness or swelling was observed at the site of implantation indicative of absence of any intra-cutaneous irritation or sensitization, respectively, in animals. Representative microscopic tissue sections of local tissues collected from around sham implants as well as around curcumin implants at 1, 4, 12, 25 and 90 days post-implantation are shown in Figure 4.10.

As is evident from Figure 4.10 (b, d, e, f, g), accumulation of mononuclear macrophages at and around the implant site was the primary cellular reaction of the body towards sham implants without any signs of contact cytotoxicity (as no necrotic sites were observed during histological evaluation). Initially from 1-4 days (Figure 4.10 (b and d)), local tissues around the sham (blank) implants showed a mild to moderate inflammatory reaction with densely-packed macrophages in addition to neutrophils and fibroblasts. At 12 days (Figure 4.10 (c)), granulation tissue formation was observed with significant increase in capillaries with reactive endothelial cells. However by 25 days (Figure 4.10 (d)), severe granulomatous reaction was observed with heavy influx of epithelioid histiocytes, multiple giant cells and fibroblasts resulting in a loosely formed granuloma by 90 days (Figure 4.10 (e)). Mild lymphocytic infiltration was observed at 25 days which did not increase any further at 90 days. These observations were consistent with other studies where implantation of hydrophobic materials like PCL was found to preferentially induce metabolically-active giant cells that are similar to surrounding macrophages (141). The observed inflammatory reaction was restricted to the site of implantation and

the surrounding layers of fibrous capsule around the implant, suggestive of only a local inflammatory response without any apparent effects at other sites distant to the site of implantation (142, 143). Furthermore, sections from liver collected at all-time points showed no histological alterations again suggestive of absence of any significant systemic toxicity.

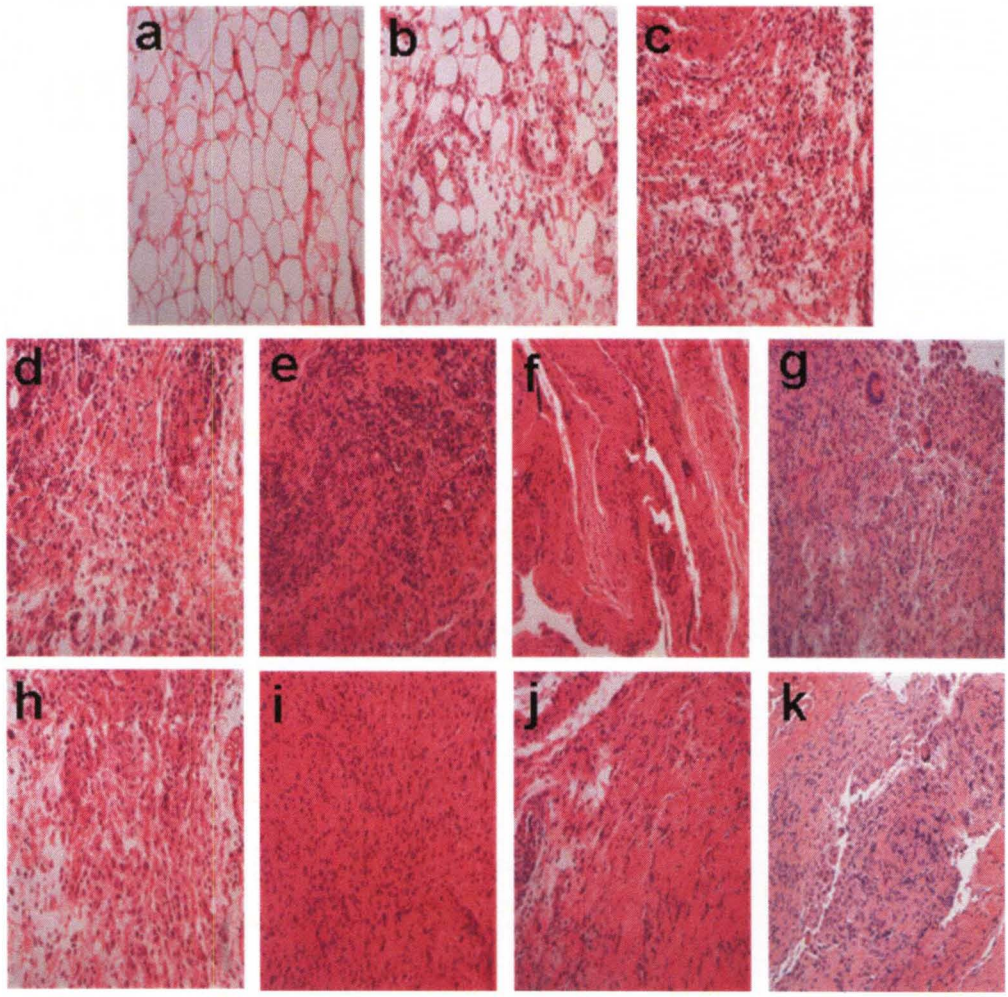


Figure 4.10. Representative histology photographs of local implantation sites after different time intervals. Panels a-c represent tissues from implantation sites of rats receiving no implant (a), sham implant (blank implants without any curcumin) (b) or curcumin implant (c) at day 1. Panels d, e, f, g represent local tissues at the site of sham implants after 4, 12, 25 and 90 days, respectively. Panels h, i, j, k represent local tissues at the site of curcumin implants after 4, 12, 25 and 90 days, respectively. Tissues from untreated animals at all the time points were similar and hence only a representative photograph is shown.

Initially, a similar reaction was observed with curcumin implants with mild to moderate inflammatory response over a period of 4 days (Figure 4.10 (c and h)) which progressed to granulation tissue formation at 12 days time period (Figure 4.10 (i)). Although a granulomatous reaction progressing to a granuloma was observed at 25 and 90 days (Figure 4.10 (j and k)), it appears that the extent of lymphocytic infiltration was lower than the sham implants. Curcumin, being a potent anti-oxidant and anti-inflammatory agent, significantly reduced the intensity of granulomatous reaction. Considering that no significant systemic toxic effects were observed during the time points investigated, and no sign of a chronic reaction was observed at later time points, it appears that the expected local reaction at implant site may not be a significant event. Further, curcumin may be countering the local reaction caused by the polymeric materials and therefore, no adverse chronic reaction can be anticipated. It may be noted that the neutrophil and lymphocyte counts of rats that received curcumin implants were comparable to that of untreated controls, particularly at the long term time point of 90 days, which reiterates the local tissue findings.

Conclusions

In summary, the subcutaneous polymeric implants investigated in this study were found to have little or no systemic toxicity but an acute inflammatory reaction at local site of implantation. Delivery of massive curcumin doses for a period of 90 days directly into the systemic circulation did not elicit any significant changes in biochemical parameters of liver and kidney function tests. Although, slight alterations in hematological parameters especially neutrophils and lymphocytes were observed but their levels were well within the normal physiological range of age-matched control animals. Over a period of 90 days, an acute local inflammatory reaction with presence of foreign body giant cells was observed with sham implants which was significantly circumvented by curcumin implants due to its anti-inflammatory properties. Absence of any significant chronic local or systemic toxicity suggests testing of these implants in a clinical setting as a viable alternative delivery system to administer curcumin (and other non-toxic natural products with therapeutic activity) directly into the systemic circulation and to determine true potential of curcumin as an anticancer/chemopreventive compound.

CHAPTER V

CHEMOPREVENTIVE/CHEMOTHERAPEUTIC EFFICACY OF CURCUMIN DELIVERED *VIA* POLYMERIC IMPLANTS

Rationale

Previous studies discussed in chapters II, III and IV showed that polymeric implants provided controlled release of curcumin into the subcutaneous tissue from where it gets absorbed into systemic circulation and gets distributed to various tissue sites. These implants were found to maintain much higher plasma, liver and brain concentrations for over a period of 3 months at ~25-30 fold lower doses as compared to dietary administration. Furthermore, these implants were also found to be safe and biocompatible when tested in ACI rats for a period of 3 months. Hematological parameters (like WBCs, RBCs, platelets, basophils, eosinophils) and biochemical parameters of liver (AST, ALP, AP, amylase) and kidney functions (Na^+, K^+, Ca^{2+}, creatinine, BUN) were found to be unaltered by continuous direct systemic administration of massive doses of curcumin by these implants. Therefore, this study was designed to further evaluate these implants for their therapeutic efficacy against estradiol (E_2)-induced mammary carcinogenesis in ACI rats and was compared to curcumin delivered *via* diet at a dose of 1,000 ppm. Furthermore, mechanistic aspects of efficacy of curcumin inthis carcinogenesis model were explored to deduce curcumin's mechanism of action against estrogen-induced mammary tumorigenesis.

Materials and Methods

Materials

Medical grade poly(ε-caprolactone) (PCL) of 121,000 molecular weight (PCL) was purchased from SurModics Pharmaceuticals (Birmingham, AL). Curcumin (C-3 complex) extracted under GMP conditions (good manufacturing practices) for human use was a generous gift from Sabinsa Corporation (East Windsor, NJ). Dichloromethane (DCM), ethoxy resorufin and pyrene was purchased from Sigma-Aldrich (St. Louis, MO). Polyethylene glycol of 8,000 molecular weight (PEG) was purchased from Fisher Scientific (Fair Lawn, New Jersey), and ethanol from Pharmco-AAPER (Louisville, KY). Medical-grade silastic tubing (3.4 mm internal diameter) was purchased from Allied Biomedical (Ventura, CA). All the materials were used as received without any further analysis.

Methods

Preparation of Implants

Implants were prepared using solvent evaporation coupled with melt extrusion technique as described previously in chapter II. Briefly, PCL and PEG (65:35) were dissolved in DCM while curcumin at a drug load of 20% was dissolved in ethanol. The two solutions were mixed together so as to prepare a homogenous solution followed by evaporation of the solvents at 70 °C to form a molecular dispersion of drug in polymer. The semi-solid mass obtained was further dried at 65 °C over night under vacuum to ensure complete removal of DCM. The molten polymer material was then extruded into silastic tubes (internal diameter 3.4 mm) at 65 °C and cut into desired lengths to form cylindrical implants.

Carcinogenesis Studies

Female ACI rats, 5-6 week-old were acclimated for 1 week under vivarium conditions, followed by administration of AIN-93M control diet. After 2 weeks, a 1.2-cm silastic implant containing 9 mg 17β-estradiol (E_2) was grafted at the back of the rats as described previously (119). All the interventions - curcumin diet (1,000 ppm), curcumin implants (two 2-cm implants, 200 mg, 20% drug load) and blank implants (PCL:PEG (65:35)) were started 4 days prior to E_2 treatment. Animals were palpated every week after 12 weeks for tumor appearance and tumor incidence was recorded in various treatment groups. The experiment was terminated when tumor incidence was >80% in E_2-treated animals (after 6 months of estrogen treatment). Animals were euthanized by asphyxiation under CO_2 and all the animals were carefully examined for the presence of mammary tumors to calculate tumor volume and multiplicity as described before (119). Blood was collected by cardiac puncture and plasma was separated by centrifugation at 3,000g. Liver was cut into small pieces, snap frozen in liquid nitrogen in cryo-vials and stored at -80°C. All the implants were also recovered, cleaned of tissue debris, dried overnight under vacuum and stored for future use to measure residual drug load.

Residual Drug Determination

The rate of release of curcumin was determined by measuring the residual curcumin in the implants recovered from animals as described in chapter III. Briefly, dried implants were dissolved in DCM:ethanol (1:1) and the solution was first diluted (1 in 10) with ethanol containing 20% DCM followed by in ethanol (1 in 40). Drug concentration was measured in diluted solution spectrophotometrically at 430 nm and the rate of curcumin release was calculated by subtracting the residual amount from initial amount per unit time.

Plasma Prolactin

Plasma prolactin was measured by using an EIA kit following manufacturer's protocol (Alpco Immunoassays, Salem, NH).

Serum Estradiol (E_2)

Serum E_2 was analyzed by using an estradiol II reagent kit purchased from Roche Diagnostics, Inc. (Indianapolis, IN) following 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 min at 4 °C to separate the nuclear content. The supernatant was further centrifuged at 11,000 g for 20 min at 4 °C to separate the mitochondria. The post-mitochondrial supernatant was then transferred to ultracentrifuge tubes and centrifuged at 100,000 g for 1 h at 4 °C to separate the microsomes. The pellet was suspended in 1 ml sucrose buffer, aliquoted and stored at -80 °C until use.

Cytochrome P450 Activities

Microsomal proteins were quantified using bicinchoninic acid (BCA) method (120) using BCA™ Protein Assay kit (Thermo Scientific, Rockford, IL). CYP1A and 1B1 activities were determined by EROD assay with and without a selective CYP1B1 inhibitor (pyrene) (Ref). UGT activity was measured using UGT-Glo™ Assay (Promega Corporation, Madison, WI) following manufacturer's protocol and CYP3A4 activity was measured using P450-Glo™ CYP3A4 Assay with Luciferin-IPA ((Promega Corporation, Madison, WI) following manufacturer's protocol but by replacing NADPH regenerating system with NADPH (5 mM).

Analysis of Plasma and Tissue Curcumin Levels

Plasma and tissues were analyzed for curcumin levels by HPLC as described in chapter III. Briefly, plasma (1.5 ml) was taken from pooled plasma of all the animals 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. Ethyl acetate from 3 extractions was pooled together and dried under vacuum. The dried residue was reconstituted in 100 µl of acetonitrile (ACN), half of which was injected to determine the plasma concentration. 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. After evaporation of ethyl acetate under vacuum, the residue was reconstituted in 1 ml ACN. The ACN solution was filtered through 0.45 µ glass-microfiber filter and evaporated again under vacuum. The residue was finally reconstituted in 100 µl ACN and half of it was analyzed by HPLC using Shimadzu liquid chromatography system equipped with LC-10ADVP pump, RF-10AXL fluorescence detector and a Shimadzu C_{18} column of 5 µm particles (250 x 4.6 mm). The three curcuminoids were separated using ACN and 1% citric acid (adjusted to pH 2.5) at a flow rate of 1 ml/min with a gradient elution in which ACN concentration was increased from 0 to 30% in first 5 min, followed by an increase to 45% in next 5 to 20 min. ACN was then maintained at this ratio till 36 min. Curcumin detection was achieved using 410 and 500 nm as excitation and emission maxima, respectively, in the fluorescence detector. Quantification of curcumin levels was done by using calibration curves prepared from pure standards of each individual curcuminoid.

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.

Results and Discussion

In our previous studies we showed that curcumin release from implants follow biphasic release pattern characterized by an initial burst release phase ranging from 7-10 days, followed by a more controlled release phase (chapter II). As shown earlier, these implants with PEG (35% w/w) released ~1.8 mg of curcumin on first day that decreased with time and released ~10.9 mg of curcumin over a period of 25 days with an average release of ~436 µg/day from each implant (Figure 5.1). The release dropped significantly by 90 days with only 19 mg cumulative curcumin being released into the systemic circulation in 3 months at a rate of ~126 µg/day/implant between 25-90 days. Since the release was expected to drop further, implants were replaced with new implants at ~138 days when average daily drug release dropped to ~95 µg/day/implant. Insertion of new implants not only provided a burst release of curcumin at the tumor initiation stage but also 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. The average daily drug release from new implants was found to be around 357 µg/day/implant from 138 to 180 days, with a total release of 36.1 mg from both old and new implants (Figure 5.1).

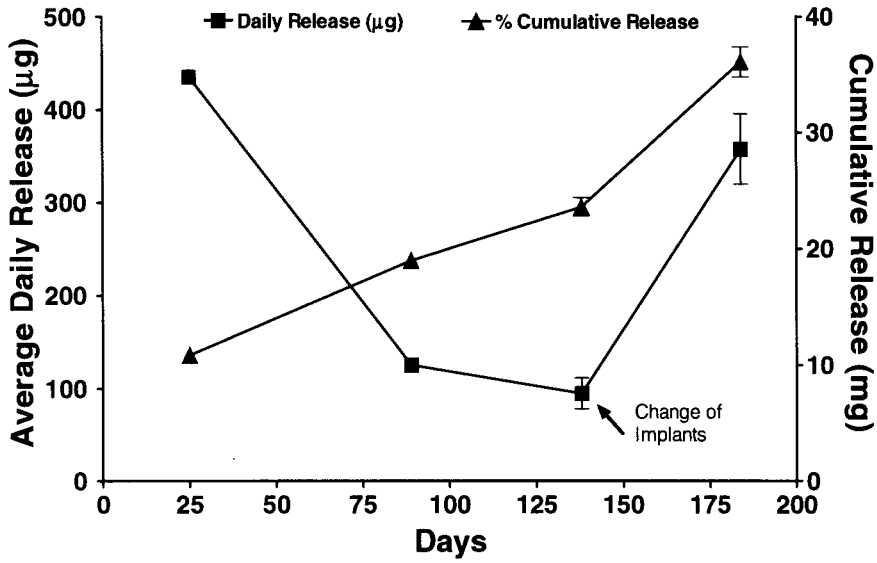


Figure 5.1. *In vivo* (daily and cumulative) release of curcumin from a 2-cm implant (200 mg, 20% drug load) recovered from animals after specific time intervals over a period of 6 months (n=3, ±SD).

Tumor Incidence

Tumor incidence was measured by palpating the mammary tissue pad of animals every week for appearance of any tumors from 12-14 weeks of E_2 treatment. The first tumor in all the E_2 treated groups appeared between 95-110 days (Figure 5.2). Although curcumin delivered *via* diet did not affect the appearance of first tumor (95 days), curcumin implants slightly delayed the tumors 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 were, therefore, changed at ~135-140 days when ~20% animals in each group developed tumors to provide a burst release of curcumin at the peak tumor initiation stage.

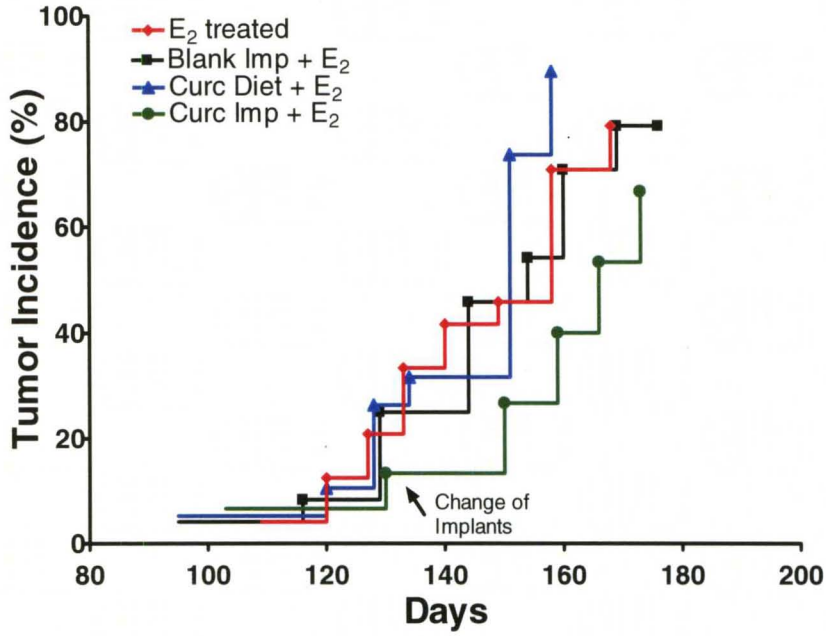


Figure 5.2. Mammary tumor incidence in female ACI rats treated with a silastic E_2 implant along with either two blank polymeric implants (2 cm, no drug), or two curcumin implants (2 cm, 20% drug load) or curcumin diet (1,000 ppm) for over a period of 6 months.

As is evident from Figure 5.2, burst release of curcumin provided by new implants at the time of initiation almost completely stopped the appearance of any new tumors for at least 3 weeks and the delay was maintained during the rest of the period. Analysis of average tumor volume per tumor (Table 5.1) and palpable tumor volume with time (Figure 5.3) further showed that after a delay of 3 weeks, the tumor growth was similar to the control groups and curcumin implants did not have any effect on growth of tumors. This observation suggests that probably curcumin had only chemopreventive activity and no chemotherapeutic potential at least in this model.

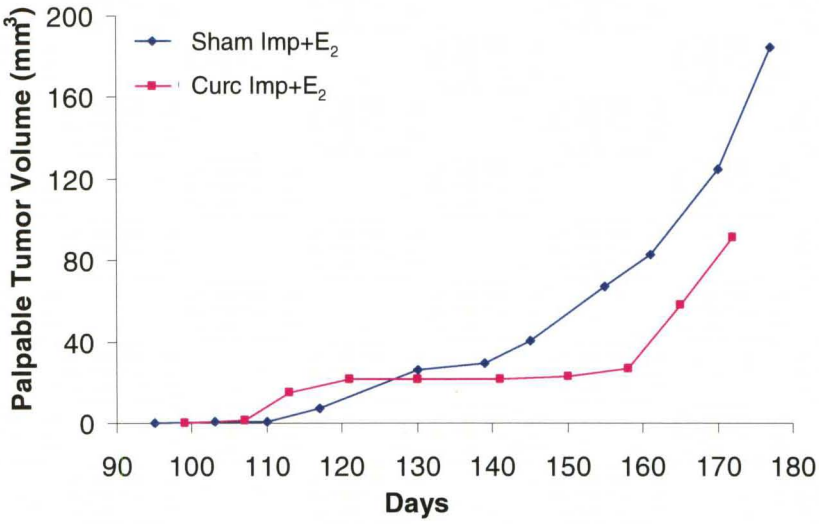


Figure 5.3. Palpable tumor growth kinetics of animals treated with E_2 along with sham implants or with curcumin implants.

With an aim to further substantiate these findings, we also measured serum prolactin levels at all the 3 time points. Prolactin is a pituitary hormone released during late pregnancy and lactation by presence of high E_2 concentrations in systemic circulation. It has also been proposed that mammary tissues produce prolactin during carcinogenesis that acts as an autocrine and paracrine growth factor for mammary tissue proliferation (144). Moreover, prolactin release from pituitary homografts have been shown to increase mammary tumor incidence in ovariectomized rats (145), suggesting a mitogenic role of prolactin in promoting the growth of already transformed normal and pre-neoplastic cells (144). It was found that at 3 weeks, curcumin implants significantly reduced plasma prolactin levels as compared to blank implants; however were ineffective at 3 months time point (Figure 5.4). Once the implants were replaced with new implants, the burst release of high curcumin doses again inhibited the E_2 mediated prolactin release at 6 months of E_2 treatment. The diet on the other hand, was ineffective at all the time points studied. These results suggest that release of bolus doses of

curcumin at 3 weeks from initial implants and at 6 months (from new implants changed after 135 days) significantly reduced the plasma prolactin levels.

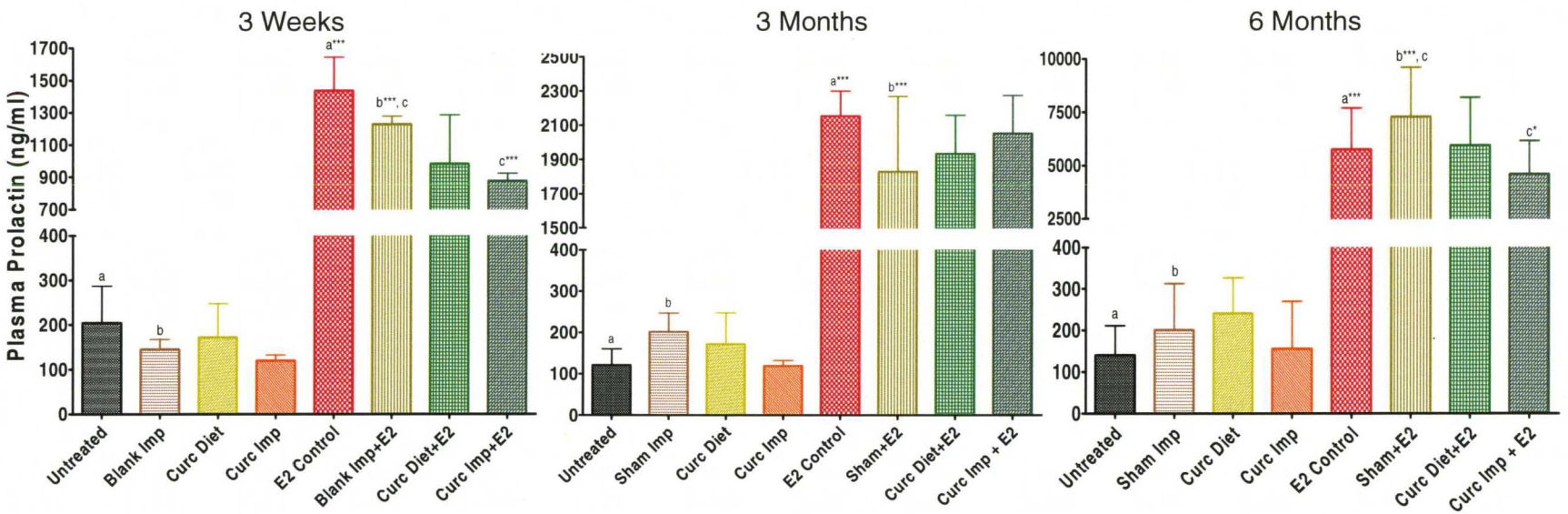


Figure 5.4. Plasma Prolactin levels of female ACI rats treated with or without a silastic E_2 implant along with either two blank polymeric implants (2 cm, no drug), or two curcumin implants (2 cm, 20% drug load) or curcumin diet (1,000 ppm) for over a period of 3 weeks, 3 Months and 6 months.

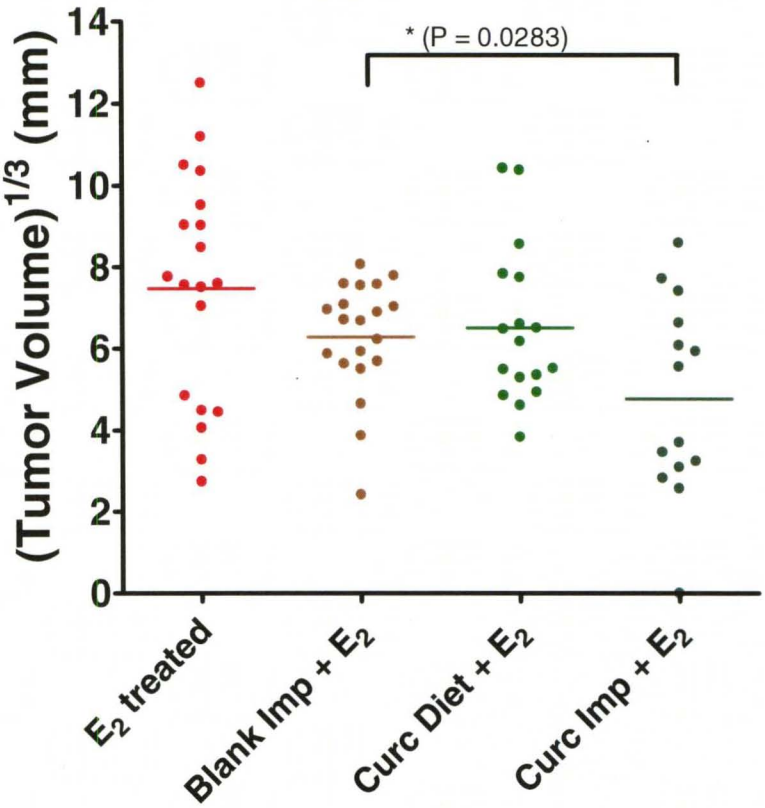


Figure 5.5. Mammary tumor volume in female ACI rats treated with a silastic E_2 implant along with either two blank polymeric implants (2 cm, no drug), or two curcumin implants (2 cm, 20% drug load) or curcumin diet (1000 ppm) for over a period of 6 months.

At the termination of the study, all tumors were measured to determine the average tumor volume and are shown in table 5.1 and Figure 5.5. Curcumin diet at a dose of 1,000 ppm was found to be ineffective as compared to E_2 treated group. However, curcumin implants were found to significantly reduce the tumor volume by ~35% as compared to blank implants with E_2 treatment group. Since, curcumin implants as such were not found to have any chemotherapeutic activity in this model; it appears that this reduction in tumor volume was due to 3 weeks delay in tumor appearance after the change of implants. Analysis of average number of tumors observed per animal (tumor multiplicity) also supported these observations (Figure 5.6 and table 5.1). Curcumin implants were highly effective in reducing the tumor multiplicity by 70% (2±1 versus 5±2)

as compared to blank implants; curcumin diet, however, was ineffective when compared with E_2 alone treatment group.

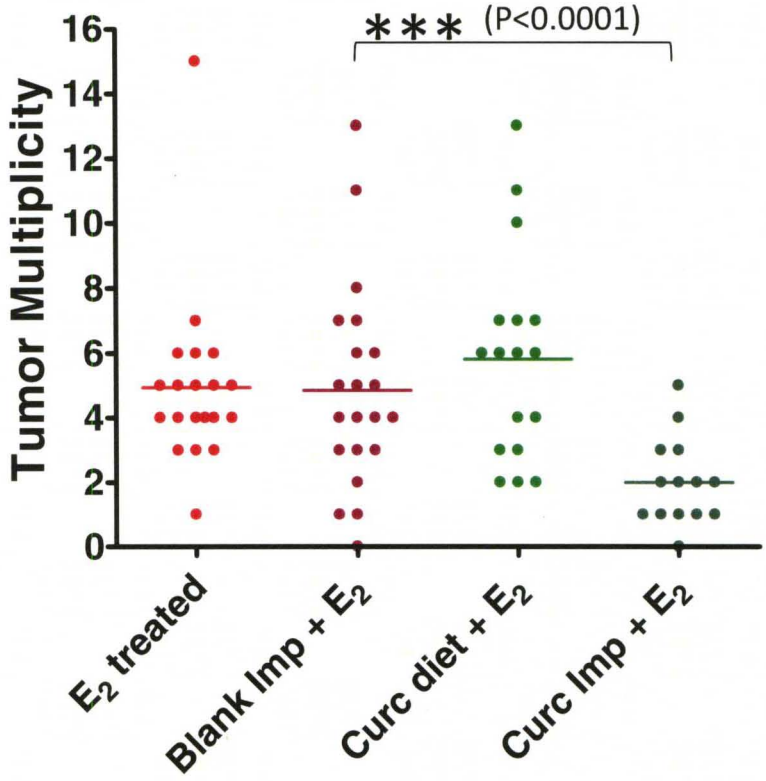


Figure 5.6. Mammary tumor multiplicity in female ACI rats treated with a silastic E_2 implant along with either two blank polymeric implants (2 cm, no drug), or two curcumin implants (2 cm, 20% drug load) or curcumin diet (1000 ppm) for over a period of 6 months.

Table 5.1.

Tumor volume and respective tumor multiplicities of animals treated with E_2 implants and either with control diet, two 2-cm blank implants, two 2-cm curcumin implants or with curcumin diet (1,000 ppm).

	E_2 treatment			Sham Imp + E_2			Curc Diet + E_2			Curc Imp + E_2		
Animal #	Tot Tum. Vol. (mm^3)	# of Tumors	Volume/ tumor (mm^3)	Tot Tum. Vol. (mm^3)	# of Tumors	Volume/ tumor (mm^3)	Tot Tum. Vol. (mm^3)	# of Tumors	Volume/ tumor (mm^3)	Tot Tum. Vol. (mm^3)	# of Tumors	Volume/ tumor (mm^3)
1	35	4	9	100	3	33	478	6	80	457	3	152
2	1954	15	130	166	7	24	147	4	37	631	1	**631***
3	1157	6	193	297	2	149	152	6	25	170	1	170
4	863	4	216	436	1	436	1111	6	185	208	3	69
5	88	3	29	470	4	118	270	3	90	222	4	56
6	66	1	66	208	5	42	286	2	143	41	2	21
7	20	3	7	57	3	19	273	3	91	29	2	15
8	349	5	70	241	11	22	119	7	17	17	1	17
9	734	4	184	325	4	81	97	7	14	22	1	22
10	113	5	23	14	4	4	113	7	16	406	2	203
11	1108	3	369	335	4	84	462	2	231	50	1	50
12	423	5	85	523	6	87	1127	2	**564***	290	5	58
13	609	5	122	354	13	27	233	10	23	34	2	17
14	431	4	108	429	5	86	166	6	28	0	0	0
15	1403	6	234	301	8	38	56	4	14			
16	737	4	184	345	1	345	624	13	48			
17	1774	5	355	177	5	35	165	11	15			
18	437	4	109	184	6	31						
19	90	6	15	433	3	144						
20	466	7	67	201	7	29						
Mean	**643**	**5**	**129**	**280**	**5**	**92**	**346**	**6**	**66**	**184**	**2**	**65**
SD	**579**	**3**	**106**	**141**	**3**	**111**	**328**	**3**	**67**	**198**	**1**	**67**

*Removed from calculations as an outlier using mean±3 S.D. relation

These results are consistent with other studies where it has been shown in DMBA (Dimethyl benz[*a*]anthracene) induced mammary cancer model that many potent chemopreventives like indole-3-carbinol (I-3C) exert maximum effect when given during pre-initiation stage (146, 147) and are more effective in reducing tumor multiplicity than tumor volume (148). This significant reduction in tumor multiplicity, therefore, again suggests that high bolus doses of curcumin delivered by new implants at the time of tumor initiation exerted maximum chemopreventive activity and probably no chemotherapeutic activity. It has also been shown in these studies that such a potent inhibition of tumor multiplicity is often due to changes in hepatic carcinogen metabolizing enzymes that are preferentially induced or inhibited to decrease the formation of reactive carcinogen metabolites and to enhance carcinogen detoxification (148). This favorable alteration in carcinogen metabolism, then ultimately results in reduced genetic mutations blunting the neoplastic trigger.

Previous mechanistic studies of estrogen induced mammary carcinogenesis have shown that E_2 mediated mammary carcinogenesis is a 2-step process (149) characterized by an initial initiation phase where highly reactive carcinogenic E_2 metabolites like 4-hydroxy estradiol (4-E_2) binds with DNA leading to genetic mutations that result in neoplastic transformation of mammary cells (Figure 5.7). Initiation phase is followed by tumor promotion phase which is mediated by hormonal activity of E_2 and 4-E_2 *via* their interaction with estrogen receptor α (ERα) and subsequent activation of proliferation signaling (150). Since curcumin implants were found to exert their effect only during the initiation stages, it appears that favorable alteration of E_2 metabolizing cytochromes played a significant role in their efficacy. E_2 is known to get metabolized by 3 distinct cytochromes giving different metabolites. E_2 metabolism by CYP1B1 results in formation of 4-OH (4-E_2) metabolites that are potent carcinogens and are initiators of

carcinogenesis cascade. On the other hand, E_2 metabolism by CYP1A (1A1 and 1A2) and CYP3A4 are true detoxification pathways forming 2-OH (2-E_2) metabolite (anticarcinogenic) and to some extent 16-OH (16-E_2) metabolite (151). Since previous studies showed that modulation of hepatic xenobiotic metabolizing enzymes affects tumor initiation and tumor incidence (146, 148), we extracted hepatic microsomes and analyzed their CYP1B1, CYP1A (1A1 and 1A2) and CYP3A4 activities (Figure 5.8a, b and c).

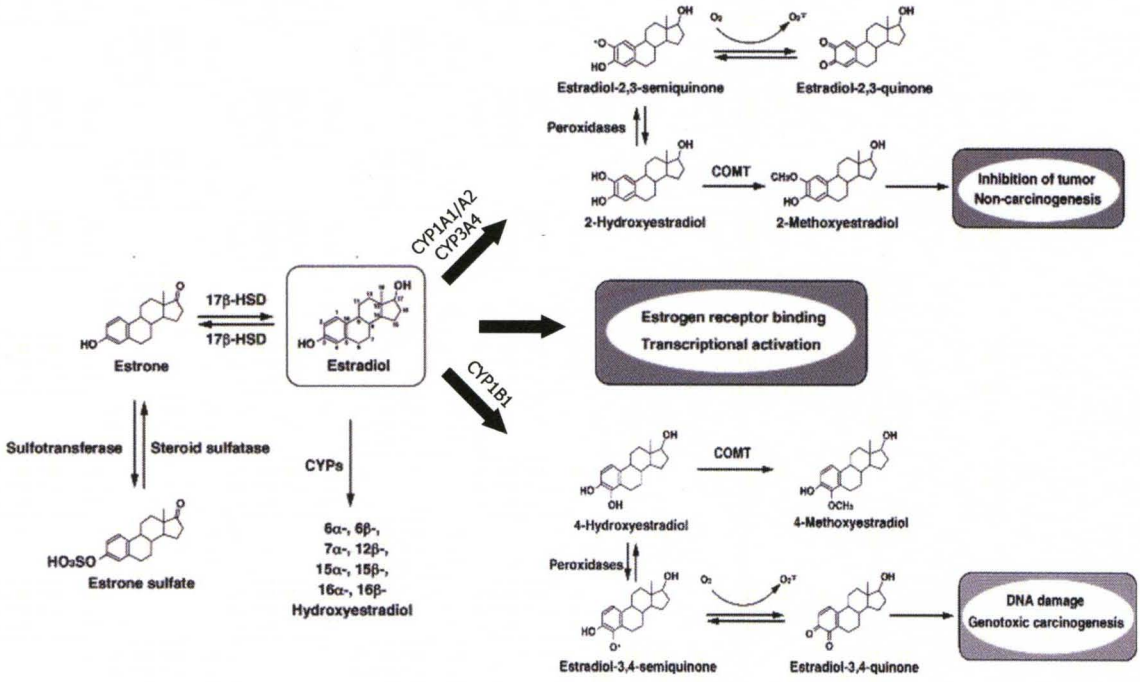


Figure 5.7. Mechanistic overview of cytochrome P450-mediated bio-activation of Estradiol to initiate carcinogenesis and to promote proliferation (modified from (152)).

CYP1B1 activity was found to exhibit time dependent kinetics with slow sustained E_2 treatment by silastic implants (Figure 5.8a). Enzyme activity increased significantly after 3 weeks of E_2 treatment which decreased to even less than untreated controls by 3 months treatment. Although, at 6 months again an increased CYP1B1 activity was observed but the increase was insignificant due to wide variation in untreated control

and E_2 treated animals. Similarly, both curcumin diet as well as curcumin implants, in the absence of E_2, increased 1B1 activity both at 3 weeks and 6 months as compared to untreated control animals. However in presence of E_2 curcumin diet either showed no effect on CYP1B1 activity (at 3 weeks and 6 months) or increased it significantly (at 3 months). Curcumin implants, on the other side, significantly reduced CYP1B1 activity at all the time points in presence of E_2. Since, CYP1B1 is known to be the major xenobiotic metabolizing enzyme responsible for formation of 4-E_2, a potent carcinogenic E_2 metabolite, inhibition of CYP1B1 activity by curcumin implants can result in reduced formation of reactive carcinogenic E_2 metabolites delaying tumor initiation and blunting tumor multiplicity.

CYP1A1 activity also showed time dependent and exposure dependent kinetics with E_2 treatment (Figure 5.8b). Enzyme activity was found to increase both at 3 weeks (though insignificantly) and 6 months (highly significantly) but significantly decreased at 3 months of E_2 treatment as compared to untreated control animals. Both curcumin diet as well as curcumin implants increased CYP1A1 activity also as compared to untreated animals in absence of E_2. However, in presence of E_2, curcumin diet did not have any effect on CYP1A1 activity at 3 weeks but increased significantly at both 3 and 6 months of E_2 treatment. Curcumin implants on the other hand, significantly increased CYP1A1 activity at 3 weeks as compared to animals with blank implants and E_2 treatment.

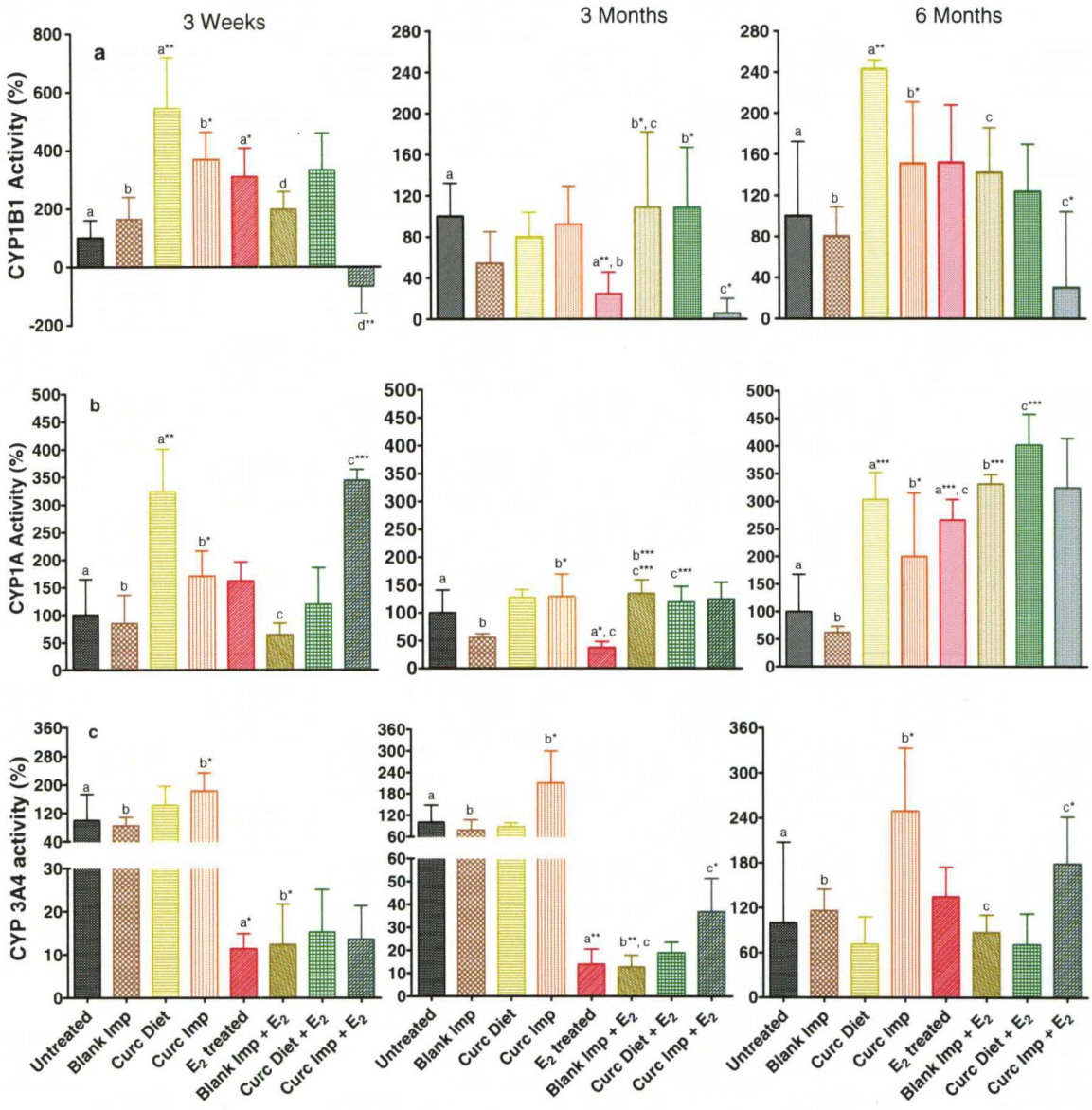


Figure 5.8. (a) CYP P450 1B1, (b) 1A (1A1 and 1A2) (c) and 3A4 activities of hepatic microsomes isolated from female ACI rats treated with or without a silastic E_2 implant along with either two blank polymeric implants (2 cm, no drug), or two curcumin implants (2 cm, 20% drug load) or curcumin diet (1,000 ppm) for over a period of 3 weeks, 3 months and 6 months.

CYP3A4 activity on treatment with E_2, significantly decreased both at 3 weeks and 3 months time point as compared to untreated animals (Figure 5.8c). Although, activity increased slightly at 6 months, due to wide variation in CYP3A4 activity in untreated

animals, significance was not achieved. Curcumin diet was not found to exert any effect on CYP3A4 activity with or without E_2 but curcumin implants significantly increased CYP3A4 activity at all the time points in absence of E_2. However, in presence of E_2 an increased activity was observed only at 3 and 6 months treatment when much higher curcumin concentrations were administered from new implants (300-400 µg/day/implant) compared to 3 months when release was low (~120 µg/day/implant).

Since, CYP3A4 is a major xenobiotic metabolizing enzyme, an increase in its activity means increased metabolism and excretion of subcutaneously delivered E_2 from the systemic circulation as non-carcinogenic metabolites and hence better therapeutic efficacy. Therefore, to gain further insights into the metabolism enhancing effects of curcumin, especially when delivered *via* implants, we measured E_2 concentration in serum both after 3 weeks and 3 months as maximum alteration in enzyme activities was observed at these time points (Figure 5.9). As is evident from the Figure 5.8, administration of E_2 by silastic implants significantly increased serum E_2 concentration at all the time points as compared to untreated control animals. Curcumin diet and implants both did not have any effect on endogenous levels of E_2 and serum estradiol concentrations were similar to untreated control animals in absence of silastic E_2 implants. However, in presence of extraneous E_2, serum E_2 concentration was found to be low with curcumin implants both at 3 weeks and 3 months, although the decrease was significant only at 3 months. Dietary curcumin, however; was ineffective.

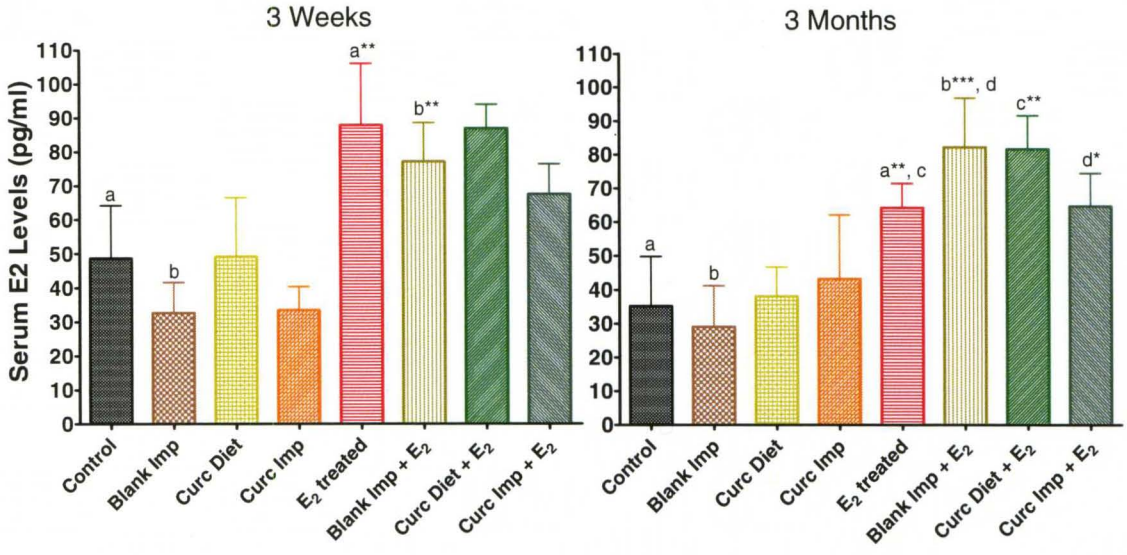


Figure 5.9. Serum estradiol (E_2) levels of female ACI rats treated with or without a silastic E_2 implant along with either two blank polymeric implants (2 cm, no drug), or two curcumin implants (2 cm, 20% drug load) or curcumin diet (1,000 ppm) for over a period of 3 weeks and 3 Months.

Since, only curcumin delivered by implants was able to modulate E_2 metabolism as compared to dietary route, we further measured curcumin levels both in plasma as well as in liver to see if these differences in activities by both routes were due to differences in curcumin concentrations (Figure 5.10). Curcumin was undetectable in plasma at all the time points when given by dietary route, consistent with other studies but was found to be 1.1, 0.3 and 0.6 nM in the case of curcumin implants in the absence of E_2. On the other hand, significantly higher concentrations of curcumin were observed in presence of extraneous E_2 as compared to untreated E_2 animals. In presence of silastic E_2 implants curcumin was detected at levels of 1.4 nM at both 3 weeks and 3 months' time point and 0.9 nM after 6 months of dietary administration and was 2.4, 1.9 and 0.9 nM after 3 weeks, 3 months and 6 months of E_2 treatment with curcumin implants. Although the plasma levels at 180 days are not significantly higher as compared to 90 days, it is to be noted that the implants were changed at 138 days, at which point the burst release

phenomenon would have occurred, to provide much higher plasma concentrations resulting in observed delay in tumor incidence and reduction in tumor multiplicity.

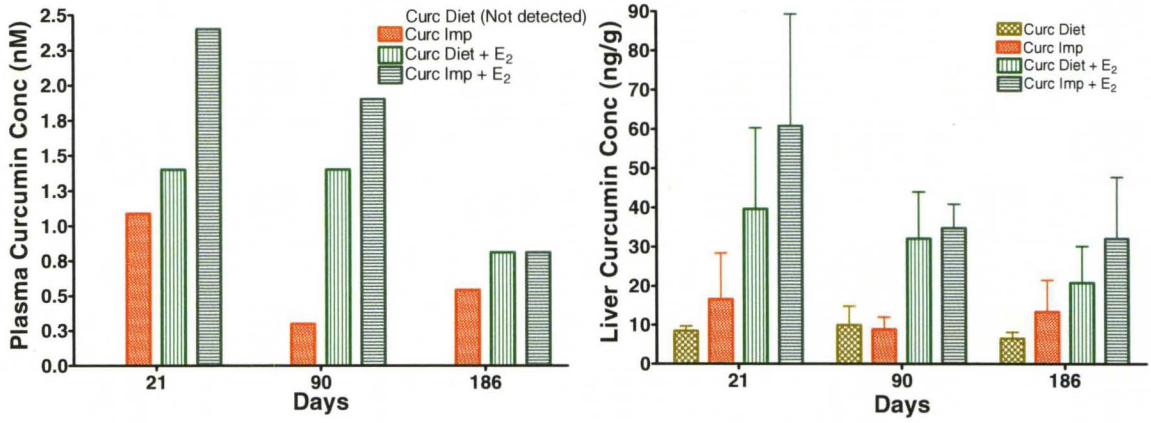


Figure 5.10. Plasma (a) and liver (b) curcumin concentration of female ACI rats treated with or without a silastic E_2 implant along with either two blank polymeric implants (2 cm, no drug), or two curcumin implants (2 cm, 20% drug load) or curcumin diet (1,000 ppm) for over a period of 3 weeks, 3 Months and 6 months.

Analysis of liver curcumin concentration also revealed a similar trend. Although curcumin was not found in plasma after dietary administration, it was found in liver tissue at all the time points even in absence of E_2 treatment. In absence of E_2, ~16.6±11.7 ng/g and 9±1 ng/g curcumin was observed by implant and dietary route, respectively, after 3 weeks. Although, almost similar levels of curcumin were observed at 3 months (9±3 ng/g) and 6 months (9±1 ng/g) by curcumin diet, with implants, the levels decreased slightly from 16.6 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 in plasma, significantly increased curcumin concentrations were observed in liver tissue in presence of E_2 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 E_2 treatment. Much higher curcumin concentrations of 61±28 ng/g, 35±6 ng/g and 32±16 ng/g tissue were observed by implant route after 3 weeks, 3 months and 6 months of E_2 treatment at

around 11-, 23- and 24-fold lower doses, respectively, compared to the dietary doses. These observations suggest that not only curcumin (at least *via* implant route) altered the hepatic metabolism of E_2 but presence of E_2 also altered curcumin metabolism to a significant extent. Curcumin is known to get metabolized by both CYP3A4 (122) and UGT (12) and it is evident from Figure 5.7c that administration of E_2 by silastic implants significantly decreased CYP3A4 activity at initial time points. Measurement of UGT activity also showed a similar decrease (Figure 5.11) both at 3 weeks and 3 months time point suggestive of decreased hepatic curcumin metabolism.

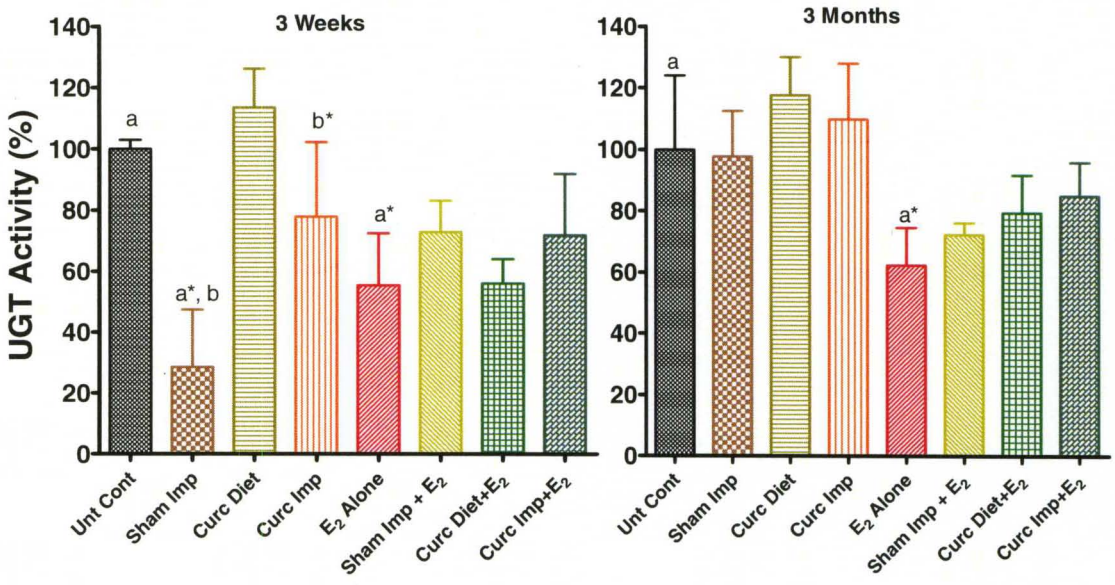


Figure 5.11. UGT activity of hepatic microsomes isolated from female ACI rats treated with or without a silastic E_2 implant along with either two blank polymeric implants (2 cm, no drug), or two curcumin implants (2 cm, 20% drug load) or curcumin diet (1,000 ppm) for over a period of 3 weeks, 3 months and 6 months.

Conclusions

Curcumin implants showed diffusion-mediated biphasic release kinetics both under *in vitro* as well as under *in vivo* conditions with a 2-fold higher drug release *in vivo* as compared to *in vitro*. Curcumin implants were able to significantly reduce the plasma prolactin levels as well as estrogen induced mammary tumor burden and tumor multiplicity as compared to curcumin diet suggestive of better chemopreventive outcomes when delivered systemically. The enhanced chemopreventive efficacy of curcumin delivered *via* implants was found to be due to favorable modulation of hepatic CYPs. CYP1B1 activity was found to be significantly reduced by curcumin implants while was increased by curcumin diet. CYP1A and CYP3A4 activities were found to be increased significantly by implant route. Taken together, these data are suggestive of increased metabolism of estradiol to carcinogenic metabolites by curcumin diet and to non-carcinogenic metabolites by curcumin implants. This differential activity was found to be due to different liver and plasma concentrations from both the routes. Curcumin concentration was found to be much higher both in plasma as well as in liver when delivered *via* implants as compared to curcumin diet.

CHAPTER VI

SUMMARY AND FUTURE PERSPECTIVES

Polymeric implants of curcumin were found to release controlled amounts of curcumin directly into the systemic circulation. The implants were found to be stable during the formulation, storage as well as under *in vivo* conditions with predictable curcumin release for up to 16 months. Plasma and brain curcumin levels were found to be significantly higher, while liver concentrations were similar, by implant delivery system compared with the dietary route despite 25-30 fold higher doses of curcumin administered orally after 3 months. Furthermore, curcumin levels achieved in liver were sufficient to alter the expression/activity of hepatic cytochromes. These Implants with chronic administration of curcumin were found to be safe and biocompatible with respect to both chronic administration of curcumin as well as to implant formulation. Curcumin doses administered by implant route were found to effectively inhibit estrogen- mediated mammary carcinogenesis in female ACI rats via favorable modulation of 17β-estradiol-metabolizing enzymes to produce non-carcinogenic metabolites. Furthermore, curcumin was not found to have any chemotherapeutic potential at these doses in this model and possessed only chemopreventive activity.

These studies showed that polymeric implants are a viable alternative for less bioavailable chemopreventive compounds like curcumin and can maintain controlled

drug levels ranging from months to years by releasing constant amounts of drug per unit time. Furthermore, this drug-delivery system can be tailored to a host of other chemopreventive and therapeutic agents thus increasing their bioavailability and reducing their effective doses as compared to traditional dietary administration. This delivery systems can also be used to screen minor phytochemicals or synthetic drugs which otherwise do not undergo activity assessment due to insufficient quantities available at the initial drug discovery stages.

Future Perspectives

- ✓ Increase drug release and rapid implant degradation: Use of alternate polymers with short half-life like PLGA can be explored to enhance the drug release with rapid rate of polymer degradation.

- ✓ These implants need to be tested using other carcinogen-induced carcinogenesis animal models or in some transgenic models with or without various chemotherapeutic compounds to determine their true potential.

- ✓ These implants could also be tested in high risk breast cancer patients so as to determine their translational potential.

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LIST OF ABBREVIATIONS

Imp	Implant
Curc	Curcumin
ECF	Extracellular Fluid
PCL	poly(ε-caprolactone)
P-15	poly(ε-caprolactone), 15,000 molecular weight
P-65	poly(ε-caprolactone), 65,000 molecular weight
DCM	Dichloromethane
ACN	Acetonitrile
PBS	Phosphate-buffered- saline
BCS	Bovine calf serum
SEM	Scanning electron microscopy
FT-IR	Fourier transform infra red spectroscopy
pXRD	Powder X-ray diffraction
DSC	Differential Scanning Calorimetry
HPCD	2-hydroxy propyl β-cyclodextrin
PEG	Polyethylene glycol, 8,000 molecular weight
I/P	Intraperitoneal
I/V	Intravenous
S/C	Subcutaneous
ACI	Augustus Copenhagen Irish
CYP1A1	Cytochrome P4501A1
CYP1B1	Cytochrome P4501A1
CYP3A4	Cytochrome P4501A1
GSTM	Glutathione S- transferase (μ)
UGT	UDP-glucuronosyltransferase
EROD	Ethoxy resorufin deethylase
E_2	17 β-Estradiol
2-E_2	2-hydroxy estradiol
4-E_2	4-hydroxy estradiol
16-E_2	16-hydroxy estradiol

CURRICULUM VITAE

Shyam Sunder Bansal
Campus Address:
580 S. Preston St.
Baxter II, Room # 320
University of Louisville
Louisville, KY-40202
s0bans01@louisville.edu, ss03bph@gmail.com
502-718-3287

A highly aspirant researcher seeking opportunity in the area of drug design/development and drug delivery to utilize my skills and to design innovative drug delivery strategies aimed to enhance drug efficacy or to achieve targetability.

Academic Profile

Ph.D.	**Pharmacology and Toxicology**
University of Louisville	Anticipated Graduation Date Aug. 2011
Master of Science	**Pharmacology and Toxicology**
University of Louisville	Louisville, KY, USA
Cum. **GPA 3.876**	2007 - 2009
Master of Science	**Pharmaceutics**
National Institute of Pharmaceutical Education and Research (NIPER)	Sector 67, SAS Nagar, Mohali, India
Cum. **GPA 9.47(/10)**	2005 – 2007
Bachelor of Science	**Pharmacy**
Delhi Institute of Pharmaceutical Sciences and Research (DIPSR)	University of Delhi, Delhi
First Division with 72.8% marks	2001-2005

Graduate Aptitude Test Examination (GATE): Indian Institute of Technology (IIT)	**Pharmaceutical Sciences** **99.94** percentile (All India Rank **6)**

Positions and Professional Memberships

Served as Graduate Student Representative for Dept. of Pharmacology & Toxicology	2010-2011
Served as president of Pharmacology & Toxicology Graduate Students Organization (PTGSO)	2010-2011
Served as a member of Graduate Affairs and Extracurricular Activities Committee	2009-2011
Member of American Association for Cancer Research	2008-Present
Pharmacist Member of Delhi Pharmacy Council, Delhi (India).	2007-Present

Honors and Awards

Recipient of **Innocentive Award** for "**Novel Ideas on New Molecular Cancer Targets for Chemotherapy**".	2011
Recipient of **Doctoral Dissertation Award** from School of Interdisciplinary and graduate studies, university of Louisville	2011
Recipient of travel award from Centre of Genetics and Molecular Medicine University of Louisville, to attend an international conference in Denmark	2009
Recipient of travel award from School of Medicine, University of Louisville to attend an international conference in Denmark.	2009
Recipient of travel award from Graduate Student Council University of Louisville, Louisville to attend an international conference in Denmark.	2009
Recipient of travel award from International Centre, University of Louisville, Louisville to attend an international conference in Denmark.	2009

IPIBS Graduate Research Fellowship, University of Louisville school of Medicine, Louisville.	2007
Achieved 2nd position in elocution competition, NIPER-India.	2005
Research Fellowship from NIPER-Punjab (India).	2005
Achieved 1st Position in science quiz competition at district level held in Delhi, India.	2000
Awarded for best science model both at state and district level held in Delhi, India.	1999

Research Projects / Thesis (Dissertation)

"To Develop and Optimize a Polymeric Implantable Drug Delivery System for Cancer Chemoprevention/Chemotherapy" for partial fulfillment of M.S. and Doctoral Candidacy in Pharmacology & Toxicology, University of Louisville, USA (**2007-present**).

(Published 3 papers out of this work, 2 ready for submission and 2 under process).

Research Supervisor: Dr. Ramesh C. Gupta

"Effect of Enthalpy Relaxation on Recrystallization Behavior of Amorphous Forms" for partial fulfillment of M.S. in Pharmaceutics (**2005-2007**).

(Published 3 papers out of this work).

Research Supervisor: Dr. Arvind K. Bansal

Research Interests

- Design and Discovery of Novel Molecular Cancer Therapeutics
- Nanotechnology in drug delivery
- Stabilization and delivery of proteins and peptides
- Design of novel formulations for poorly bioavailable drugs
- Solid State properties of drugs (crystalline and amorphous) and their polymorphs

Technical Skills

Analytical Skills:

Well versed with analytical techniques like High performance liquid chromatography (HPLC) for drug determination in plasma and tissues, UV-visible/fluorescence spectrophotometry, differential scanning calorimetry (DSC), FT-IR spectroscopy, Scanning Electron Microscopy and working knowledge of GC-MS, Powder X-ray diffraction.

Molecular Biology Techniques

Cell culture, Western Blotting, Histochemistry, ELISA, EIA, microsomal activity assays (CYP1A1, CYP1B1, CYP3A4, UGT), GSH analysis etc. Well experienced in animals studies, their planning execution and collection of organs like liver, lung, kidneys, ovaries, intestine, mammary tissue, brain, uterus, pituitary gland etc.

Selected Peer Reviewed Publications

Bansal, S.S., Goel, M., Aqil, F., Vadhanam, M.V., Gupta, R.C. Advanced Drug Delivery Systems of Curcumin for Cancer Chemoprevention (**In press**).

Bansal, S.S., Kausar, H., Aqil, F., Jeyabalan, J., Vadhanam, M.V., Ravoori, S., Gupta, R.C. An implantable formulation of Curcumin: Safety and Biocompatibility. Drug Deliv Transl Res 2011 1(4):332-341.

Bansal, S.S., Vadhanam, M.V., Gupta, R.C. Development and In Vitro-In Vivo Evaluation of Polymeric Implants for Continuous Systemic Delivery of Curcumin. Pharm Res 2011 28(5):1121-1130

Bansal, S.S., Kaushal A.M., Bansal A.K. Enthalpy Relaxation Studies Of Two Structurally Related Amorphous Drugs And Their Binary Dispersions. Drug Dev Ind Pharm 2010 36(11):1271-1280

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Bansal, S.S., Kaushal, A. M., Bansal A.K. Molecular and Thermodynamic Aspects of Solubility Advantage from Solid Dispersions. Mol Pharm. 2007 4(5):794-802.

Bansal, S.S., Joshi, A., Bansal, A.K. New Dosage Formulations to Target Delivery of COX-2 Inhibitors. Drugs Aging 2007 24(6):441-51.

Joshi, A., **Bansal, S.S.**, Hiwale, P., Bansal, A.K. Amorphous Drug Delivery. Indian Pharma Business Compendium. 2007: 33-36.

Datta, P., **Bansal, S.S.**, Verma, S., Kumar, N. Pulmonary Drug delivery: A Platform for Difficult to Deliver Drugs. CRIPS. 2005 6, 10-15.

Papers Under Consideration/Preparation

Bansal, S.S., Kausar, H., Vadhanam, M.V., Ravoori, S., Gupta, R.C. Polymeric Implants Improve Tissue Distribution of Curcumin as Compared to Oral Administration over Extended Periods of Time (submitted).

Bansal, S.S., Kausar, H., Vadhanam, M.V., Jeyabalan, J., Aqil, F., Ravoori, S., Gupta, R.C. Curcumin Implants, not curcumin diet are effective against estrogen induced mammary tumors in ACI rats (manuscript ready for submission).

Bansal, S.S., Jeyabalan, J., Vadhanam, M.V., Gupta, R.C. Effect of Sustained, Low-Dose Curcumin on Benzo[*a*]Pyrene-Induced Tissue DNA Adducts in Rat Model (manuscript under preparation).

Bansal, S.S., Aqil, F., Gupta, R.C. Effect of piperine on metabolism and tissue distribution of curcumin delivered *via* Implants (manuscript under preparation).

Abstracts and Poster Presentations

Bansal, S.S., Kausar, H., Vadhanam, M.V., Jeyabalan, J., Aqil, F., Rai, S.N., Ravoori, S., Gupta, R.C. Curcumin implants, not curcumin diet inhibits estrogen induced-mammary carcinogenesis. **In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 02-06; Orlando, FL, Abstract # 1863.**

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Gupta, R.C., **Bansal, S.S.,** Aqil, F., Cao, P., Jeyabalan, J., Russel, G.K., Ravoori, S., Vadhanam, M.V. 2009. A novel concept in delivering chemopreventive compounds. **In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, CO. Philadelphia (PA), Abstract # 944.**

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Bioavailability. **In: Proceedings of the 58th Indian Pharmaceutical Congress; 2006 Dec 1-3; Mumbai, India;** AP-195, pp155-156.

Personal Profile

Nationality: **Indian**

Fluent Languages: **English, Hindi & Punjabi**