3,3'-Diindolylmethane Encapsulated Chitosan Nanoparticles Accelerates Inflammatory Markers, ER/PR, Glycoprotein and Mast Cells Population During Chemical Carcinogen Induced Mammary Cancer in Rats

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Abstract The present study aimed to investigate the effect of 3,3'-diindolylmethane (DIM) on inflammatory markers, estrogen receptors (ER), progesterone receptors (PR), level of glycoprotein and the mast cell population in 7,12-dimethylbenz (a) anthracene (DMBA) 25 mg/kg b.wt. induced rat mammary carcinogenesis. After 8 weeks of tumor formation, rats had access to an oral administered with DIM 10 mg/kg b.wt. and DIM@CS-NP 0.5 mg/kg body weight respectively for 8 weeks. The oral administration of DIM@CS-NP 0.5 mg/kg b.wt. suppressed the Cox-2, NF- κ B and TNF- α protein expression on DMBA induced rats compared to DIM 10 mg/kg b.wt. The ER/PR levels were increased on DMBA induced rats, treated with DIM@CS-NP 0.5 mg/kg b.wt. reduced ER/PR level as well as glycoprotein and mast cell population than DIM 10 mg/kg b.wt. The result shows that, DIM@CS-NP 0.5 mg/kg b.wt. has the potentially inhibit abnormal levels of inflammatory markers, ER, PR, levels of glycoprotein and mast cell population compared to DIM 10 mg/kg b.wt.

Keywords Breast cancer · DIM@CS-NP · NF- κ B · TNF- α · Glycoprotein

Introduction

3,3'-Diindolylmethane (DIM) is a dietary indole compound naturally present in glucosinolate conjugates in *Brassica* vegetables, have been broadly studied for its anticancer activity both in vitro and in vivo models. Due to low solubility/high lipophilicity of DIM has an extremely poor oral bioavailability is a foremost barrier to working with a potential pharmaceutical agent [1]. Previous studies indicate that, the encapsulation of 3,3'-diindolylmethane in zein/carboxymethyl chitosan nanoparticles is an assured approach to get better their stability adjacent to crucial condition and deliver controlled release manner [2]. Such approaches made it possible to develop a great interest in determining the encapsulation of DIM in nanoparticles could prove a gene level approach.

Many pathological situations, including cancer, are characterized by an imbalance between survival and apoptosis signals. The tumor necrosis factor-alpha (TNF- α) is also critical to cellular communication during host defense, inflammation and organogenesis. TNF receptor activation leads to recruitment of intracellular adaptor proteins that activate multiple signal transduction pathways [3, 4]. Moreover, The default pathway is the induction of genes involved in inflammation and cell survival, the ligand binding to TNFR1 induces a range of inflammatory mediators and growth factors via activation of the AP-1 transcription factors or IK β kinase that, in turn, activate nuclear transcription factor- κ B (NF- κ B) [4].

COX-2, NF- κ B and TNF- α levels amalgamates plays a very important role in cell proliferation. Further, signal transduction pathway of TNF- α , prompt and begin downstream actions lead to apoptosis and NF- κ B activation [5]. Furthermore, the NF- κ B signaling pathways plays critical task in controlling cell proliferation, survival, tumor

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invasion, metastasis, drug resistance and stress response. In non-stimulating circumstances, NF- κ B is sequestered in the cytoplasm through taut connection with NF- κ B inhibitory-protein I κ B, subsequent stimulation, including cytokine binding to its receptor, activation of I κ B kinase (IKK) complex. Conventionally, these may lead to phosphorylation and following degradation of the inhibitory-protein I κ B, allow NF- κ B to translocate into the nucleus and binds to its target DNA to direct the expression of NF- κ B target genes [6]. In addition, cyclooxygenase 2 (COX-2) is an inducible enzyme and is activated by extracellular stimuli such as growth factors and pro inflammatory cytokines [7]. It is a pleiotropic enzyme that, mediates many physiological functions such as, inhibition of cell apoptosis, augmentation of angiogenesis and increased cell motility [8].

Commonly, estrogen and progesterone hormones control numerous functions of the common and irregular mammary epithelium, including cell growth and development. The majority of the activities of estrogens and progesterone are interceded by means of particular steroid receptors, and one would expect that multiplying cells ought to contain estrogen receptors (ER) or potentially progesterone receptors (PR).

Earlier, in our laboratory, we studied the oxidant and anti-oxidant status, lipid profile of DIM@CS-NP on DMBA induced mammary carcinogenesis [9]. Here, the study we have scrutinized to notably unveil whether DIM@CS-NP can alter inflammatory signalling pathway, ER/PR, glycoprotein level and mast cell population.

Materials and Methods

Chemicals

7,12-Dimethylbenz(a)anthracene (DMBA), 3,3'-diindolylmethane (DIM), chitosan and sodium tripolyphosphate (TPP) were purchased from Sigma (St. Louis, MO, USA). The primary antibodies used in this study were procured from Santa Cruz Biotech, USA. Other chemicals were analytical grade and were procured from Sigma Aldrich Chemicals Pvt. Ltd.

Preparation and Characterization of DIM@CS-NP

DIM@CS-NP was synthesized by ionic gelation method used TPP as a gelating agent [10, 11]. Characterized by UV, Zeta Sizer, Particle size analyzer, SEM analysis, FT-IR analysis, Thermogravimetric analysis (TG), in vitro drug release and pharmacokinetic study.

Rats Handling

Female Sprague-dawley rats 6–7 weeks old (weighed 130–150 g), were purchased from National Institute of Nutrition, Hyderabad, India. Rats were maintained under controlled conditions of temperature ($24 \pm 2^\circ\text{C}$), humidity ($50 \pm 10\%$) and 12 h light/dark cycle and bare access to standard food and water provided in the Central Rat House, Rajah Muthiah Medical College and Hospital, Annamalai University, Chidambaram, Tamil Nadu, India. This study was approved by the Institutional Animal Ethics Committee (IAEC), regulated by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) (Reg. No. 160/1999/CPCSEA and Proposal No. 1123). An acclimatization period of at least 1 week was allowed before initiating the experimental protocol.

Tumor Induction

DMBA was used as a carcinogen for the present study, mammary tumor was induced by a single subcutaneous injection of 25 mg/kg body weight of DMBA dissolved in 1 mL emulsion of sunflower oil (0.75 mL) and physiological saline (0.25 mL) [12].

Experimental Design

The rats were divided into six groups with six rats in each group ($n = 6$). Group 1 rats served as control. Groups 2–rats were received 25 mg/kg b.wt. of DMBA during the first week of the experiment. The groups 3 and 4 were treated with different doses of DIM, DIM@CS-NP (10, 0.5 mg/kg b.wt.) respectively for every alternative day. Groups 5 and 6 were received DIM 10 mg/kg b.wt. in 0.5% of DMSO and DIM@CS-NP 0.5 mg/kg b.wt. and served as drug control. The doses were fixed, based on a dose response study that we already examined in our lab [9]. Rats were weighed weekly once, until the end of the experimental period of 16 weeks, after rats were euthanized by cervical decapitation. A portion of mammary tissue homogenized in 0.1 M Tris–HCl buffer, pH 7.4 (10% homogenate) and used for molecular experiments.

Western Blotting

Western blotting was carried out to evaluate the expression pattern of COX-2, NF- κ B and TNF- α proteins by using the method of Laemmli [13]. The mammary tissue samples were homogenized with a buffer [5 mM sodium azide, 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride

(PMSF), 10 mM NaHCO₃ (pH 7.0)]. The homogenate was centrifuged at 12,000×*g* for 30 min at 4 °C to remove debris. Sample containing 50 mg of total cellular proteins was loaded and separated using 10% SDS polyacrylamide gel electrophoresis. The resolved proteins were blot transferred onto a PVDF membrane (Millipore). The membranes were incubated with the blocking buffer containing 5% w/v non-fat dry milk and then incubated with the primary antibody in 10 mL of antibody-diluted buffer (Tris-buffered saline and 0.05% Tween-20 with 5% milk) with gentle shaking at 4 °C for 8–12 h. After this, membranes were incubated with their corresponding secondary antibodies (anti rabbit and anti-mouse IgG conjugated to horseradish peroxidase) for 2 h at room temperature. Membranes were washed thrice with TBST for 30 min. After extensive washing with TBST, the bands were visualized by treating the membranes with 3,3'-diaminobenzidine tetra hydrochloride (Western blot detection reagent). Bands were scanned using a scanner and quantified by Image J, a public Java Image processing software, Wayne Rasband, NIH, Bethesda, MD, USA. Percentage of expression was calculated by keeping expression of protein in control rats as 100%.

Pathological Studies

5 mm sections of the paraffin-embedded tissues were cut using a microtome and then rehydrated with xylene and graded series of ethanol. The specimens were then stained accordingly as mentioned below. Histopathological analysis of mast cells by Toluidine blue staining was carried out by the method of Migliaccio et al. [14]. Glycoprotein content in the mammary tissues was stained with Periodic Acid Schiff (PAS) base, according to the method of Yamabayashi [15].

Immunohistochemical (IHC) Expression

Immunohistochemical analysis was performed on 5 mm paraffin embedded mammary tissue section on poly-L-lysine coated glass slides. The tissue slides were deparaffinized by placing the slides in an oven at 60 °C for 10 min and then rinsed twice in xylene for 5 min each. The slides were then hydrated in a graded ethanol series for 10 min each and then finally washed in double distilled water for 5 min. The sections were incubated with 1% H₂O₂ in double distilled water for 15 min at 22 °C, to quench the endogenous peroxidase activity, then the sections were rinsed with Tris-HCl containing 150 mM NaCl (pH 7.4) and blocked with blocking buffer 1× TBS, 0.05% Tween 20, 5% NFDM for 1 h at 22 °C. After washing with 1× TBS containing 0.05% Tween 20, the sections were incubated with primary antibodies, anti-estrogen receptor, anti progesterone receptor overnight at 4 °C, followed by

incubation with the respective secondary antibodies IgG-HRP conjugates for 1 h at 4 °C. After washing with 1× TBS containing 0.05% Tween 20, the immune reactivity was developed with 0.01% DAB and H₂O₂ for 1–3 min and the sections were observed (40 x) for brown color formation under bright field in a microscope.

Statistical Analysis

Statistical analysis was performed using SPSS 17 (SPSS, Inc., Chicago) statistical package. The data are expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA) followed by Duncan multiple range test (DMRT) comparison method was used to correlate the difference between the variables. Data are considered statistically significant if *p* values are less than 0.05.

Results

Western Blotting Analysis in Mammary Tissues of Control and Experimental Rats

Figure 1 illustrated the molecular protein changes occurred during tumorigenesis. We sought to determine the inflammatory proteins by investigating some important regulators involved in the pathways. COX-2, NF-κB and TNF-α proteins were found to be significantly increased in

Fig. 1 **A** Representative blot analysis of inflammatory protein expression COX-2, NF-κB and TNF-α in mammary tissue. 1 Control, 2 DMBA, 3 DMBA + DIM, 4 DMBA + DIM@CS-NP, 5 DIM, 6 DIM@CS-NP. **B** Band intensities were scanned by the densitometer. Histograms of densitometric analysis represent the ratio of COX-2, NF-κB and TNF-α expression. Values that do not share a common superscript in the same column differ significantly at *p* < 0.05 (DMRT). Statistical significance was compared within the groups as follows: control (a), DMBA (b), DMBA + DIM (c), DMBA + DIM@CS-NP (d), DIM (a), DIM@CS-NP (a)

DMBA induced (2) tumor bearing rats when compared to control rats. Whereas, the levels of these proteins were found to be significantly decreased during treatment with DIM 10 mg/kg b.wt. (3) and DIM@CS-NP 0.5 mg/kg b.wt. (4) treated rats, when compared to tumor bearing rats. On the other hand, in our findings showed that, DIM@CS-NP 0.5 mg/kg b.wt. was more effective than DIM 10 mg/kg b.wt. However, there were no significant changes in the expression of these proteins in DIM 10 mg/kg b.wt. (5) and DIM@CS-NP 0.5 mg/kg b.wt. (6) alone treated rats when compared to control (1) rats.

Effect of DIM and DIM@CS-NP on Mast Cell Population

Figure 2a–f shows the effect of DIM and DIM@CS-NP on the histopathology analysis by toluidine blue staining respectively. The DMBA induced (B) rats shows showed

significantly increased number of mast cell population in the mammary tissues. Here, DIM@CS-NP 0.5 mg/kg b.wt. (D) treatment significantly reduces the number of mast cell population level than DIM mg/kg b.wt. (C). However, DIM (E) and DIM@CS-NP (F) alone treated groups of rats, no significant changes were absorbed compared to control (A).

Effect of DIM and DIM@CS-NP on Glycoprotein

Figure 3 represented the Periodic Acid Schiff (PAS) staining shows the glycoprotein level in the mammary tissues of control and experimental rats. The DMBA induced (B) rats shows excess accumulation of glycoprotein in the mammary tissues. Here, DIM@CS-NP 0.5 mg/kg b.wt. (D) treatment significantly reduced the glycoprotein level than DIM 10 mg/kg b.wt. (C). However, DIM (E) and DIM@CS-NP (F) alone treated groups of rats; no significant changes were absorbed control (A).

Fig. 2 The effect of DIM and DIM@CS-NP on the histopathological analysis of (a–f) toluidine blue staining in mammary tissue of control and experimental rats. The control and drug control (DIM and DIM@CS-NP) rats (a, e and f) showed normal mammary tissue. The DMBA (b) induced rats showed increased mast cell accumulation

when compared with control rats. However, oral administration of DIM@CS-NP (d) treated rats showed diminished mast cell accumulation as compared to DIM (c) administration, no significant changes were absorbed in DIM and DIM@CS-NP ($\times 40$ magnifications)

Fig. 3 The effect of DIM and DIM@CS-NP on the histopathological analysis of PAS staining (a–f) respectively in mammary tissue of control and experimental rats. The control and drug control (DIM and DIM@CS-NP) rats (a, e and f) showed normal mammary tissue. The DMBA induced rats (b) showed increased glycoprotein when

compared with control rats. However, oral administration of DIM@CS-NP (d) treated rats showed diminished area of glycoprotein levels as compared to DIM (c) administration rats. $\times 40$ magnifications, no significant changes were absorbed in DIM and DIM@CS-NP

Immunohistochemical Expression of ER in Mammary Tissues of Control and Experimental Rats

Figure 4 represented ER expressions in mammary tissues of control and experimental group of rats are represented. (A–F) shows the ER expression, Control (A), DIM (E) and DIM@CS-NP (F) alone treated rats showed normal expression of ER, whereas, DMBA induced (B) rats showed higher expression of the hormone receptor. However, the administration of DIM@CS-NP 0.5 mg/kg b.wt. treatment to tumor bearing rats significantly decreased the expression of the hormone receptor as compared to DIM 10 mg/kg b.wt. According to these findings, it clearly shows the chemotherapeutic effect of DIM@CS-NP.

Immunohistochemical Expression of PR in Mammary Tissues of Control and Experimental Rats

Figure 5 represented PR expressions in mammary tissues of control and experimental group of rats are represented. (a–f) shows the PR expression. Control, DIM and DIM@CS-NP (a, e and f) alone treated rats showed normal expression of PR whereas, DMBA induced (B) rats showed higher level expression of the hormone receptor. However, the administration of DIM@CS-NP 0.5 mg/kg b.wt (d). treatment to tumor bearing rats significantly decreased the expression of the hormone receptors as compared to DIM 10 mg/kg b.wt (c).

Fig. 4 The effect of DIM and DIM@CS-NP on the histochemical analysis of ER (a–f) respectively in mammary tissue of control and experimental rats. The control and drug control rats (a, e and f) showed normal mammary tissue staining, the DMBA induced rats

(b) showed increased expression of ER when compared with control rats. However, oral administration of DIM@CS-NP (d) treated rats showed diminished area of expression of ER levels as compared to DIM (c) administration ($\times 40$ magnifications)

Discussion

Nanoscale drug release preparation from biocompatible and biodegradable polymers comprises a developing approach to drug delivery and tumor targeting. The limitations of current therapy offer a compelling rationale for the development of surrogate modalities for the targeted delivery for the treatment of solid tumors [16]. An exhilarating possible solution in cancer treatment is to encapsulate the drug in a biocompatible material that can be injected into the blood stream with the purpose of delivering drug to a tumor and killed tumor cells [17]. Defects in the cascade of apoptosis related events during neoplastic development could affect the apoptotic cell death and disrupt homeostasis regulation in the mammary tissues. Thus, the pathological status of tumorigenesis was identified by altering the cell morphology and tissue architecture. In the present study, we have elucidated the drug targeted action of DIM@CS-NP scrutinized inflammatory markers.

In mammary carcinogenesis, apoptosis was mediated by numerous molecules for instance, tumor necrosis factor- α and Caspases. Indeed, the TNF- α exerts cytolytic or cytostatic action against tumor cells, TNF- α also responsible for a signaling gives number of events, including activation of transcription factor and apoptosis [18, 19].

Moreover, DIM modulates downstream signaling pathways impinge on proliferation, angiogenesis and apoptosis. These pathways have been shown to be inhibited both in vitro and in vivo by DIM. Previous result was found to be that DIM, and the formulated B-DIM treatment, could inactivate NF- κ B and DNA binding activity in breast tissues [20]. In humans and experimental rats prostaglandins are believed to play a key role in the development and progression of many cancers [21]. Furthermore, cyclooxygenase inhibition of COX-2 isozyme blocking the prostaglandin (PG) cascade may have a consequence on neoplastic growth and

Fig. 5 The effect of DIM and DIM@CS-NP on the histochemical analysis of PR (a–f) respectively in mammary tissue of control and experimental rats. The control and drug control (DIM and DIM@CS-NP) rats (a, e and f) showed normal mammary tissue staining, the DMBA induced rats (b) showed increased expression of PR when

compared with control rats. However, DIM and DIM@CS-NP (c and d) treated rats showed decreased expression of PR in the mammary tissues. However, oral administration of DIM@CS-NP treated rats showed diminished the expression of PR as compared to DIM administration ($\times 40$ magnifications)

development by inhibiting explosion of angiogenesis and metastasis [22]. Based on the previous report in both clinical and experimental studies, found that COX-2 may be a crucial factor in the development of mammary gland carcinogenesis [23]. In our study, we observed that, an increased expression of COX 2, NF- κ B and TNF- α in mammary tissue of DMBA induced rats. It is evidenced from the earlier literature that the mRNA expression of inflammatory mediators in colons of DSS-induced colitis, altered the COX-2 and TNF- α -level in treated with DIM 20 mg/kg b.wt. [24]. Our results also in line with these findings the expression of COX 2, NF- κ B and TNF- α found to be increased in DMBA induced positive control group. Notably, DIM treated group showed slender decrease of COX 2, NF- κ B and TNF- α expression, however DIM@CS-NP supplemented group markedly decreased the expression of COX 2, NF- κ B and TNF- α compared to DMBA induced positive control group which indicates suppression of tumor development.

Glycogen is the storeroom form of glucose in cells and is vital for energy deliver and glucose homeostasis. In cancer metabolism and the metabolic reprogramming during the adaptive process of cells within the tumor microenvironment and resistance to anticancer therapies have been well documented [25]. Our finding shows increased glycogen content in the mammary tissues of DMBA induced rats, the DIM@CS-NP treatment significantly reduced the glycogen than DIM. Many tumors contain mast cells (MCs), further, it is a part of the innate immune system and is taking on to and activated in the microenvironment of a developing tumor. The accumulation of MCs has been associated with enhanced growth and invasion of several human cancers [26]. On the other hand, MC penetration has been connected with superior prognosis in breast carcinoma [27]. Our result shows the increased mast cell number in the breast tissues of DMBA-induced rats and the DIM@CS-NP treatment significantly reduced the mast cell population than DIM.

Hormonal receptors are distributed in the human body, mainly in the female reproductive organs but also in non-reproductive organs as well. There the ER and PR levels vary in different tissues. Their levels are also influenced by factors such as age, obesity, and existence of cancer. The endogenous estrogen 17 β -estradiol can be metabolized to 16 α -hydroxyestrone (16 α OHE1) or 2-hydroxyestrone (2OHE1). In contrast to 2OHE1, 16 α OHE1 is highly estrogenic and has been found to enhance the proliferation of estrogen-sensitive breast cancer cells in culture [28]. Therefore, IHC analysis of DMBA-induced tumor bearing rats showed elevated levels of ER and PR expression, which indicates development of hormone-dependent mammary carcinoma. However, oral supplementation of DIM@CS-NP in tumor bearing rats showed down regulated expression of ER and PR status compared to DIM. Moreover, our result was also collaborating with previous studies of deepalakshmi et al., who also postulated that the POEet in DMBA induced mammary carcinoma rats by exemplify the changes of ER and PR status of mammary tissues [29].

Conclusion

The insolubility in aqueous solutions, short half-life and low bioavailability of DIM following oral administration presented a significant challenge in developing an effective delivery system for its use as a chemotherapeutic agent. However, DIM@CS-NP significantly alters the expression of COX 2, NF- κ B and TNF- α on DMBA induced rats and reduce ER/PR, mast cell population and glycoprotein level.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflict of interests.

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