

## Inhibition of phospholipase A2 expression by the *trans, trans* conjugated linoleic acid isomers in mammary tumorigenesis induced by N-methyl-N-nitrosourea in Sprague Dawley Rat

Mohammad A. Islam<sup>1\*</sup>, Sohel Hasan<sup>1</sup>, S. Munira<sup>1</sup>, M Sohanur Rahman<sup>1</sup>,  
M. Asaduzzaman<sup>1</sup>, M. Muedur Rahman<sup>1</sup>, M Khatun<sup>1</sup>, M.H Khan<sup>1</sup>, Ziaul Amin<sup>2</sup>  
Md Rezaul Karim-2,<sup>1</sup>and Matiar Rahman<sup>1</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh.

<sup>2</sup>Department of Genetic Engineering and Biotechnology, Jessore University of Science and Technology, Bangladesh.

**Corresponding author:** Professor Dr. Mohammad Amirul Islam, Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh. Fax: +88-0721-750064, Tel: +88-0721-750049/4109, E-mail: [maislam06@gmail.com](mailto:maislam06@gmail.com)

**Abstract:** The ultimate purpose of this study was to assess that the *t,t* CLA (*trans trans* Conjugated Linoleic Acid) is a powerful anticancer agent in a number of tumor model systems. Here, we report that a mixture of *t,t* CLA isomers was able to significantly inhibited mammary tumorigenesis with the reduction of phospholipase A2 (PLA2) and cyclooxygenase-2 (COX-2) expression levels referenced with *c9,t11* CLA, *t10,c12* CLA, and Linoleic Acid (LA). Female Sprague Dawley rats were intraperitoneally injected with MNU and were subjected to five diets groups for 16 weeks. COX-2 and *cPLA*<sub>2</sub> are associated with cancer cell survival and tumor angiogenesis, consequently arachidonic acid itself is a strong apoptotic signal that may facilitate cancer cell death. We determined the cytoplasmic PLA2, COX-2 and PPAR- $\gamma$  expression levels in a panel of rat mammary tumors by western blotting analysis and checked the apoptosis index by TUNEL assay. The *t,t* CLA treatment considerably decreased, in the level of cytoplasmic PLA2 and COX-2 expression by the inhibition of arachidonic acid metabolism as well as prostaglandin compare with other CLA isomers and control. The *t,t* CLA significantly, increased apoptosis compared with the references. In the treatment of *t,t* CLA, inhibition of *cPLA*<sub>2</sub> resulted in a down regulation of PPAR- $\gamma$  activity as compared with others references. Our data strongly support the model in which mammary cancer growth is favored when intracellular arachidonic acid levels are suppressed by inhibition of *cPLA*<sub>2</sub> and COX-2.

**Running title:** Inhibition of tumorigenesis by *t,t* CLA.

**Abbreviations:** Conjugated Linoleic Acid (CLA), Arachidonic Acid (AA), Phospholipase A (PLA), Cyclooxygenase (COX).

**Keywords:** Conjugated Linoleic Acid isomers; *t,t* CLA isomer; Mammary tumorigenesis, MNU

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### I. Introduction

Conjugated linoleic acid (CLA) consists of a complex mixture of geometrical (*cis* and *trans*) and positional isomers in the fat of ruminant animals which have been shown to have anti-mutagenic and anti-carcinogenic activities[1,2]. The major isomers in dairy animals are *cis*-9, *trans*-11, *trans*-10, *cis*-12, *trans*-9, *trans*-11 and *trans*-10, *trans*-12 [3]. These isomers, however, appear to have different mechanisms of action and physiological effects on human health. A mixture of *trans, trans* CLA (designate *t, t* CLA) isomer has also been shown to be a potent cancer-preventative agent in human cell line[3,4,5,6]. The incorporation of *t,t* CLA in the membrane phospholipids also reduced the arachidonic acid content relative to that of *t10,c12* CLA and *c9,t11* CLA, and this result may inhibit the biosynthesis of PGE<sub>2</sub> from arachidonic acid via the cyclooxygenase and prostaglandin H synthase pathways. There is emergent evidence that COX-2 and its key metabolite, prostaglandin E2 (PGE<sub>2</sub>), a major eicosanoid produced within the intestine, are associated with enhanced cell proliferation and survival [7]. An important source of arachidonic acid for COX-2 is a ubiquitously expressed family of phospholipases (PLA). This complex gene family consists of eight secretory and three cytoplasmic phospholipases [8]. The *cPLA*<sub>2</sub> plays a central role in cytokine-induced release of arachidonic acid [9]. In cells stimulated by tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), *cPLA*<sub>2</sub> undergoes immediate activation via mitogen-activated protein kinase-mediated phosphorylation and Ca<sup>2+</sup>-directed translocation from the cytoplasm to the

endoplasmic reticulum and nuclear membrane[10,11]. At the endoplasmic reticulum, it hydrolyzes phospholipids to arachidonic acid, which in turn are metabolized by the actions of co-localized COXs to produce prostaglandin (PG) metabolites [12].

In addition, [13] Scorrano recently showed that Ca<sup>2+</sup> released from the endoplasmic reticulum via arachidonic acid is a key mechanism for controlling apoptotic cell death. In contrast, PGs, especially PGE<sub>2</sub>, have been reported to possess potent proliferative and tumorigenic capacity. To identify the central role of cPLA<sub>2</sub> and COX-2 in maintaining the balance of arachidonic acid and PG levels, the coordinated regulation of these two proteins may be a critical mechanism for maintaining the balance between proliferation and apoptosis. Mr Liu and his colleagues also reported that the c9, t11 and t10, c12 CLA isomers inhibit the formation of arachidonic acid and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which are involved in cell growth [14]. Much attention has not been paid to the antiproliferative effects of the mixture of t,t conjugated linoleic acid isomers with double bonds at C7,C9; C9,C11; C10,C12; and C11,C13 (designate t,t CLA) on mammary tumors. We recently found that t,t CLA inhibited the growth of an osteosarcoma cell line, MG-63, and breast cancer cell line, MCF-7, in a dose-dependent manner [15,4]. Meanwhile, no reports are available on the growth inhibitory effect of t,t CLA in mammary tumorigenesis induced by N-methyl-N-nitrosourea in SD rat by the inhibition of expression of cPLA<sub>2</sub>, COX-2 levels. Despite the minimal expression of cPLA<sub>2</sub>, COX-2 levels were distinctly increased with concomitant overproduction of PGE<sub>2</sub> [16]. We hypothesized that the t,t CLA coordinated regulation between cPLA<sub>2</sub> and COX-2 expression may attenuate the apoptotic signals that are mediated by arachidonic acid while further potentiating the proliferative signals elicited by PGE<sub>2</sub>. Never the less, to further explore the potential for coordinated regulation of cPLA<sub>2</sub> and COX-2 expression in mammary tumors were analyzed for cPLA<sub>2</sub> and COX-2 expression and correlated with apoptotic index.

## II. Materials and methods

### Materials

Linoleic acid (99.0%), N-methyl-N-nitrosourea (NMU), Tween 20, skim milk, Triton X-100, leupeptin, pepstatin, Sodium dodecylsulfate (SDS) and N, N, N', N'-tetramethylethylenediamine (TEMED) were obtained from Sigma Chemical Company (St. Louis, Mo). Ammonium persulfate and mercaptoethanol were purchased from Amresco (Solon, OH). i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology, INC. (Seongnam, Korea). Rabbit polyclonal cPLA<sub>2</sub> and COX-2 antibodies, and anti-rabbit and mouse IgG-horse-radish peroxidases were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-β-actin was purchased from Sigma Chemical Co. (St. Louis, MO). Vectastain ABC kits were purchased from Vector Laboratories Inc. (Burlingame, CA). All other reagents used were of analytical grade.

### Preparation of CLA isomers

Synthetic CLA was prepared from linoleic acid by alkaline isomerization and methylated with 1.0 N H<sub>2</sub>SO<sub>4</sub>/methanol [17]. The methyl esters of c9, t11 CLA and t10, c12 CLA isomers were prepared from synthetic CLA methyl ester by crystallization in conjunction with urea treatment [18]. The t,t CLA methyl ester was prepared from synthetic CLA methyl ester according to the method described by [19]. The free forms of c9, t11, t10, c12 and t, t CLA isomer samples were prepared from their methyl esters by the conventional method of acid hydrolysis [18]. The purity of CLA isomer samples was found to be 88.2% for c9,t11 CLA, 97.8% for t10,c12 CLA, and 98.4% for t, t CLA when analyzed by gas chromatography (GC) [20].

### Animal Experiment with NMU

Pathogen-free female Sprague Dawley rats, 7 weeks of age, were purchased from Samtako Bio Korea Inc. (Osan, Korea). Animals were housed in polycarbonate cages (one rat/cage) in a temperature (22 ± 2 °C) and humidity (55 ± 5%)-controlled facility with a specific pathogenic free (SPF) and a 12 h light/dark cycle systems, and fed the basal AIN93G diet (Table 1). One week later, animals were randomized by body weight and divided into Control, 1% linoleic acid, 1% c9, t11 CLA, 1% t10, c12 CLA, and 1% t,t CLA treatment groups (6 rats/group), and given an i.p. injection of MNU at a single dose of 50 mg/kg of body weight, followed by subjecting their diets (Table 1) until 17 weeks. Diet and water were ad libitum. Beginning with the initiation of tumor and continuing thereafter, body weight and food intake were measured weekly. All rats were sacrificed 18 weeks after the mammary tumor initiation.

### Mammary tumor analysis

Tumors weight, size and numbers were recorded from rats sacrificed at the termination of animal experiment. All mammary tumors, trimmed off debris, of each treatment group were longitudinally excised into two sections and saved for further analysis. One section was fixed immediately in 10% phosphate-buffer formalin (pH 7.3) for histological examination and TUNEL assay, and the rest of them were kept in deep freezer (Ultra Low Temp. Freezer, KDF-400L) at -71 °C until use for other biochemical analysis.

### **Western blotting**

The mammary tumors were homogenized in RIPA buffer (150 mM NaCl, 0.1 % NP40, 0.5 % deoxycholic acid, 0.1 % SDS, 50 mM Tris, pH 7.4) containing protease inhibitors, 21  $\mu$ M leupeptin and 15  $\mu$ M pepstatin [3]. Homogenates were centrifuged at 13000 rpm for 5 min at 4 °C and the supernatant collected as protein content sample. Protein content was determined by Bradford Protein Assay Protocol [21]. Assessment of cPLA2, COX-2 and PPAR-gama were performed by Western blotting as described by [22, 3, 23]. Bound antibodies were detected with the aid of an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham Biosciences, Buckingham, U.K.). The relative protein levels were determined using the Gel logic 100 Imaging System(EastmanKodakCo.USA)

### **Determination of Phospholipids**

Total lipid of mammary tumor was extracted by[24]. Tumor (about 600 mg) was taken into the centrifuge test tube (20 mL, teflon) containing 10 mL chloroform: methanol (2:1, v/v) and 50  $\mu$ l of 10% BHA solution. The test tube was homogenized with Polytron for one min and centrifuged for 5 min at 5,000 rpm. The upper layer (methanol) was removed and the bottom layer (chloroform containing lipid extract) was recovered and washed three times with 10 mL of 0.47 M Na<sub>2</sub>SO<sub>4</sub> by hand shaking for 30 sec and then dried over Na<sub>2</sub>SO<sub>4</sub> anhydrous. Total lipid was weighed after removing the solvent by Eyer rotary evaporator (Tyko, Japan). Then 10 mg of total lipid was loaded in the silica Maxi-Clean™ cartridge for isolation of PL. After removing cholesterol ester, triacylglycerol, non-esterified fatty acids, monoacylglycerol and diacylglycerol fractions from the column, the PL fraction was eluted with 20 mL MTBE/methanol/ ammonium acetate (pH 8.6; 10:4:1, v/v/v) as described by [25] Hamilton and Comai K. Each fraction was dried under nitrogen to use a sample. PL sample was methylated according to the method of [17] Park. Composition of fatty acids, including CLA isomers, was analyzed by GC (Hewlett Packard 5890) equipped with FID and a fused silica capillary Supelcowax-10 column (60 m x 0.32 mm, i.d., 25  $\mu$ m film thickness). Oven temperature was increased from 180°C to 200°C at a rate of 2°C/min and then held for 50 min. Injection port and detector temperatures were 240 and 260°C, respectively. Nitrogen (99.9%) was used as a carrier gas with a flow rate of 2 mL/min. The fatty acids of the samples were identified through comparison with the relative retention time of the standards.

### **Statistical analysis**

Statistical analysis was carried out using analysis of variance followed by Duncan's Multiple Range Test. Mean differences with P<0.05 were considered statistically significant.

## **III. Results**

### **Histology of tumors from rats treated with t,t CLA**

The microscopic analysis of tumors stained with hematoxylin and eosin are shown in **Figure 1**. The tumors in control rats were all adenocarcinomas (**Figure 1-K**). The lesions were rather cellular and consisted of a proliferation of epithelial and stromal components; the epithelial elements ranged from reactive to malignant in nature. The majority of the tumor is adenomatous and exhibits features of florid sclerosing adenosis admixed with low-grade ductal carcinoma in situ. Focal areas exhibited features diagnostic of adenocarcinoma, including increased mitotic index, atypical mitotic figures, moderate to severe cytologic atypia, coagulative tumor cell necrosis, and jagged infiltrating margins. The lesions were well circumscribed in areas and associated with a brisk host response composed of reactive stromal cells and a mixed inflammatory infiltrate. Angiogenesis is much more prominent than in the adenomas in the supplemented rats.

In contrast, the tumors from t,t CLA-treated rats showed that the epithelial cells were exhibited mild to moderate cytological atypical and low mitotic index, and lesion was well circumscribed and exhibited prominent papillary architecture (**Figure 1-O**). In the tumors from rats treated with c9, t11 CLA, t10, c12 CLA and linoleic acid (**Figure 1-L-N**), the lesion was moderately cellular and consisted of epithelial and stromal components. Inclusively, the cytoarchitectural features were indicative of a fibroepithelial lesion, such as a fibroadenoma. The stromal and vascular proliferation was much less than that seen in the adenocarcinoma of tumors from control rats.

### **Induction of apoptosis in tumors from rats treated with t,t CLA**

Apoptosis is responsible for the inhibition of MNU-induced mammary tumorigenesis by t,t CLA as shown in **Figure 2**. To test this hypothesis, the apoptosis was determined by TUNEL (**Figure 2-B-F**), which are considered hallmarks of apoptosis, for the mammary tumor tissues from rats treated with t,t CLA, with references to c9,t11 CLA, t10,c12 CLA and linoleic acid. TUNEL analysis showed more frequent occurrence of darkly stained TUNEL-positive nuclei in the mammary epithelium of the t,t CLA treated-rats than the occurrence in the mammary epithelium of the control, c9,t11 CLA, t10,c12 CLA and linoleic acid-treated rats (**Figure 2-B-F**). Apoptotic cell index (AI) was found to be 6.74% in control rats, and 8.38% in linoleic acid-

25.40% in c9,t11 CLA-, 30.46% in t10,c12 CLA-, and 38.53% in t,t CLA-treated rats (Figure 4G), which was significantly higher,  $p < 0.05$ , in the tumors from t,t CLA-treated rats than that in the tumors from c9,t11 CLA, t10,c12 CLA and linoleic acid-treated rats. Based on these results from TUNNEL the induction of apoptosis by t,t CLA was greater than that of c9,t11 CLA, t10,c12 CLA, and linoleic acid.

#### **The t,t CLA isomer reduced the expression of PLA2, COX-2 and PPAR-gamma**

Mammalian cells contain diverse forms of PLA2 which is highly specific for arachidonic acid (AA) and participates in the release of this fatty acid in various cell types. In the present investigation, the expression of PLA2 and COX-2 was determined to establish the relation of inhibition in MNU-induced mammary tumorigenesis by Western blotting assays. The expression of PLA2 protein was significantly decreased ( $p < 0.05$ ) in the tumors from t,t CLA-treated rats, relative to that of t10,c12 CLA and c9,t11 CLA-treated rats (Figure 4). In contrast to the PLA2 protein, the expression level of COX-2 significantly,  $p < 0.05$ , reduced in t,t CLA-treated mammary tumors, relative to that of c9,t11 CLA, t10,c12 CLA (Figure 5). As a result, the expression of COX-2 protein down-regulates the mammary tumorigenesis induced by MNU; t,t CLA-induced inhibition in MNU-induced mammary tumorigenesis is associated with a reduced PLA2 protein level as well as COX-2 protein level.

To understand the mechanism how cPLA2 directly contribute to the induction of AA metabolism in the regulation of PPAR-gamma mediated gene, we first examined the possible effect of cPLA2 on PPAR activation. The PPAR-gamma protein expression was significantly decreased ( $p < 0.05$ ) in the tumors from t,t CLA-treated rats, relative to that of t10,c12 CLA and c9,t11 CLA-treated rats (Figure 6). As a consequence there of the t,t CLA-treated rats with cPLA2 down expression would likely have decreased production of AA and eicosanoid in the nuclei for PPAR inactivation.

#### **Incorporation of CLA in phospholipid fraction**

Phospholipases A2 represented a diverse group of enzymes that catalyze the hydrolysis of ester bonds at the sn-2 position of membrane phospholipids and release fatty acids like arachidonic acid or linoleic acid. The alteration of mammary tumors phospholipids from rats treated with t,t CLA relative to c9,t11 CLA, t10,c12 CLA, and linoleic acid was examined as shown in Figure 3. AA concentrations of PL was lower in the t,t CLA treatment. No significant difference between treatment groups and vehicle control group was observed for AA concentration in female SD rats as well as the differences in this fatty acids were not statistically different in all dietary groups. Likewise, the CLA feedings did not alter the proportion of other fatty acids in any substantial way regardless of the differences of CLA isomers in the diets at termination time.

### **IV. Discussion**

It was reported previously that t,t CLA inhibits the growth of osteosarcoma cell line, MG-63 and breast cancer cell line, MCF-7, in a dose-dependent manner[3, 4]. Now, we are going to bring to light that t,t CLA inhibits the mammary tumorigenesis in rats induced by MNU. Although a mixture of CLA isomers, and individual isomers of c9,t11 CLA and t10,c12 CLA were extensively studied for their anticarcinogenicity in several animal models, no report on the anticarcinogenicity of t,t CLA in animal models, especially rat mammary carcinogenesis are available so far to the best of our knowledge. To find out this important mode of action of t,t CLA, a series of experiments were performed. In the present study, rat mammary carcinogenesis initiated by MNU, the inhibitory efficacy of t,t CLA was superior to that of c9, t11 CLA and t10,c12 CLA isomers, which might be associated with a inactivation of PLA2 and COX-2 proteins to reduced arachidonic acid metabolism. The key outcome of the current study is that t,t CLA isomers inhibit cPLA2 and COX-2 induced eicosanoid released from rat mammary tumors, which is strongly supported by the findings from similar studies using macrophages or endothelial cells[11,13,14]. The reduction in eicosanoid production by CLA in the present study might be due, at least in part, to the observed reduction of arachidonic acid, which serves as the main substrate for the biosynthesis of prostaglandins, in rat mammary tumors total lipids following treatment with CLA. It has been reported that t,t CLA competes with other CLA isomers and linoleic acid for the incorporation into membrane phospholipids but also that CLA interferes with the production of arachidonic acid from linoleic acid [26] resulting in a reduced arachidonate pool and subsequently reduced eicosanoid production.

PLA2 is functionally linked to COX-2 within the perinuclear membrane and is the primary source of arachidonic acid production for COX-2 [27, 26]. It is still unknown, whether CLA isomers affect tumorigenesis in rat in mammary gland. Diverse PLA2 enzymes hydrolyze the phospholipids releasing fatty acids, mainly AA. Its further metabolism of AA by COX-2 gives rise to prostaglandin. Never the less, the results and figures obtained from this study showed that t,t CLA inhibit PLA2 and COX-2 expression and reduced formation of AA compared with the CLA isomers. Several studies previously reported showed that CLAs reduce the formation of eicosanoids in various animal cells[22, 3]. The results of Eder's study suggest that CLA isomers

reduce availability of AA in endothelial cells due to reduced activity of sPLA2, which results in diminished formation of eicosanoids [24].

Never the less, the results of this study strongly suggest that CLA may change the activity of phospholipases in macrophage cells as well. It was observed that both CLA isomers (cis-9, trans-11 and trans-10, cis-12) inhibited the total activity of PLA2 (measured as AA concentration) (**Figure 1**) without the change in the expression mRNA of cPLA2 (**Figure 3**). Additionally trans-10, cis-12 CLA (not cis-9, trans-11) isomer reduced the activity and mRNA expression of sPLA2 in macrophages (**Figure 2**). In endothelial cells both isomers of CLA at 50  $\mu$ M reduced gene expression of the sPLA2 [24], but in other studies trans-10, cis-12 CLA was more bioactive than cis-9, trans-11 CLA isomer. This isomer reduced expression and activity of many enzymes e.g. COX-2, stearoyl-CoA desaturase or LPL lipase [6, 23].

In the physiological conditions cPLA2 is required for the regulation of sPLA2 activity and the process involves the metabolites of lipoxygenase-1 (LOX) [1]. These findings suggest that AA derivatives may play a role in the regulation of sPLA2 expression in cell. The hypothetical link might involve the limitation of availability of AA and linoleic acids for oxygenases and decreased synthesis of their derivatives. Similar phenomena were observed in murine fibroblasts, where inhibitors of LOX caused suppression of sPLA2 induction [3]. Thus, CLA may indirectly inhibit sPLA2 induction, but the details of the mechanism remain to be determined.

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**Table 1.** Composition of diets used in this study

Ingredient <sup>1</sup>	Diet (g/100 g diet) <sup>2</sup>				
	Control	LA	CT	TC	TT
Casein	20	20	20	20	20
Corn oil	5.0	4.8	4.8	4.8	4.8
Dextrose	65	65	65	65	65
AIN-76 mineral	3.5	3.5	3.5	3.5	3.5
AIN-76A vitamin mix	1.0	1.0	1.0	1.0	1.0
Alphacel	5.0	5.0	5.0	5.0	5.0
Methionone	0.3	0.3	0.3	0.3	0.3
Cholin bitartrate	0.2	0.2	0.2	0.2	0.2
Linoleic acid	0	1.0	0	0	0
c,t CLA	0	0	1.0	0	0
t,c CLA	0	0	0	1.0	0
t,t CLA	0	0	0	0	1.0
Total	100	100	100	100	100

\*<sup>1</sup>Diet was prepared as described, which contained the amount of ingredients such as casein, corn oil, dextrose, AIN-76 mineral, AIN-76A vitamin mix, alphacel, methionone, and cholin bitartrate. The purity of linoleic acid, c9,t11 CLA, t10,c12 CLA and t,t CLA isomers provided was 99.0, 89.5, 99 and 99.0% respectively, and thus, amounts added were adjusted accordingly.

\*<sup>2</sup> Diet groups were as control, linoleic acid (LA), c9, t11 CLA (CT), t10,c12 CLA (TC) and t,t CLA (TT).

### Legends of Figure

**Figure 1:** t,t CLAs reduce mammary tumors induced by MNU in S D rats. The MNU, LA, CT, TC, and TT represent control, linoleic acid, c9, t11 CLA, t10, c12 CLA, and t,t CLA, respectively. Photograph of mammary tumors bearing rats of (A) MNU, (B) LA, (C) CT, (D) TC and (E) TT groups were i.p. injected with single dose of MNU (50 mg/Kg body weight). (K-O) Light microscopical photographs of MNU-induced mammary tumors in SD rats by CLA. Panel identification: (K) MNU alone, (L) LA, (M) c9, t11 CLA, (N) t10, c12 CLA (O), and t,t CLAs (I). The bars represent 4µm (x 200).

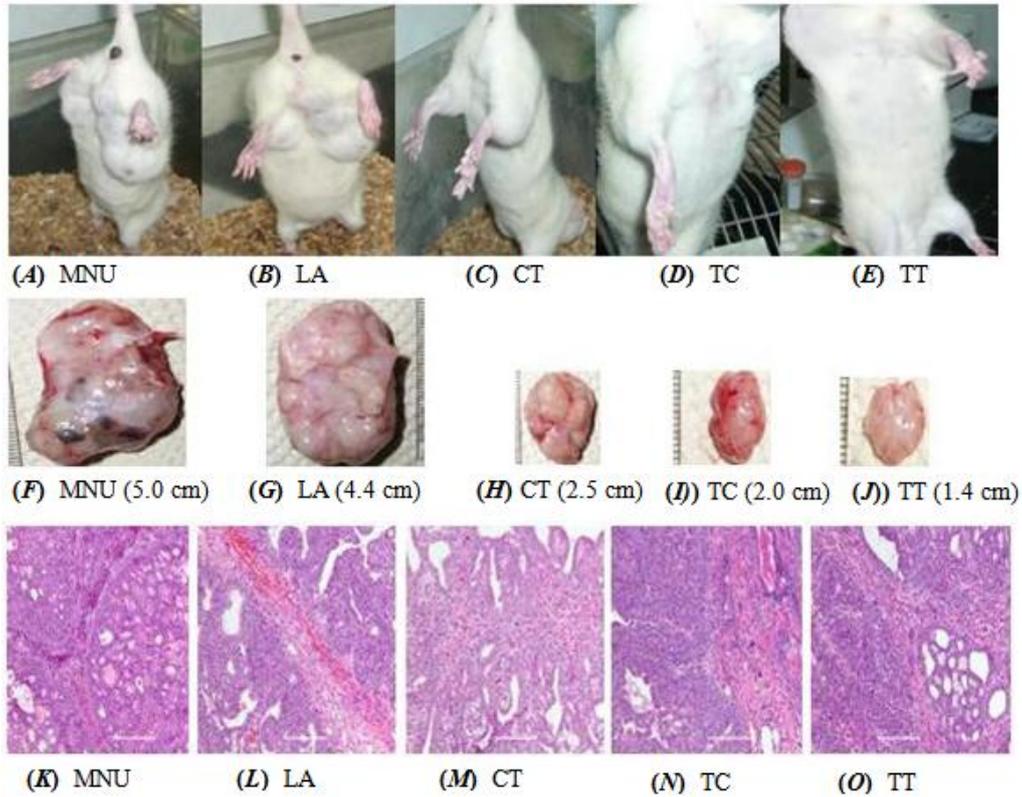
**Figure 2:** The MNU, LA, CT, TC, and TT represent control, linoleic acid, c9,t11 CLA, t10,c12 CLA, and t,t CLA, respectively. In situ detection of apoptotic cell death of mammary tumor treated with CLA isomers. Dark brown nuclei were positive cells. Panel identification: treatment of MNU alone (A), LA (B), c9,t11 CLA (C), t10,c12 CLA (D), and t,t CLA (E). The bars represent 4µm (x 200). (F) The apoptotic index (%), determined by TUNEL assay.

**Figure 3:** t,t CLAs reduces the arachidonic acid production by inhibition of PLA2 in MNU-induced rat mammary tumorigenesis. Procedure was described in materials and methods. The MNU, LA, CT, TC, and TT represent control, linoleic acid, c9, t11 CLA, t10,c12 CLA, and t,t CLA, respectively. Data are expressed as means ± SD (n = 3). Means with different small letters are significantly different at p < 0.05

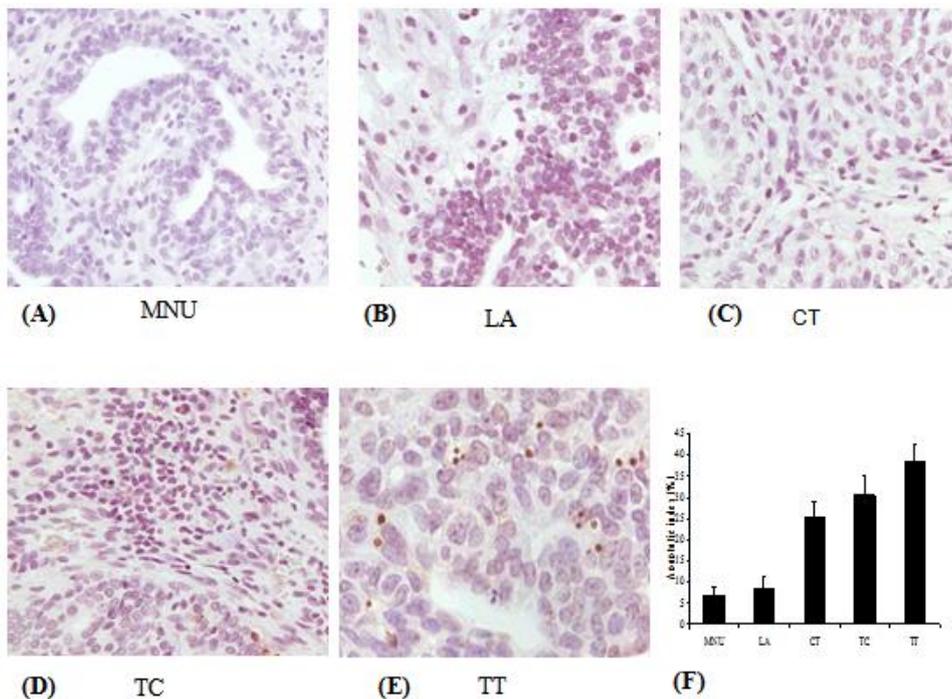
**Figure 4:** cPLA<sub>2</sub> protein level was determined MNU-induced rat mammary tumors by western blot analysis. It displayed that t,t CLAs reduced the expression of cPLA-2 expression level reference with linoleic acid, c9,t11 CLA and t10,c12 CLA. The MNU, LA, CT, TC, and TT represent control, linoleic acid, c9, t11 CLA, t10, c12 CLA, and t,t CLA, respectively. The band intensities relative to the control were quantified. Values are means ± SD (n = 3). Means with different small letters are significantly different at p < 0.05

**Figure 5:** COX-2 protein level was determined MNU-induced rat mammary tumors by western blotting analysis. Remarkably, t,t CLAs reduced the expression of COX-2 expression level reference with linoleic acid, c9,t11 CLA and t10,c12 CLA. The MNU, LA, CT, TC, and TT represent control, linoleic acid, c9, t11 CLA, t10, c12 CLA, and t,t CLA, respectively. The band intensities relative to the control were quantified. Values are means ± SD (n = 3). Means with different small letters are significantly different at p < 0.05

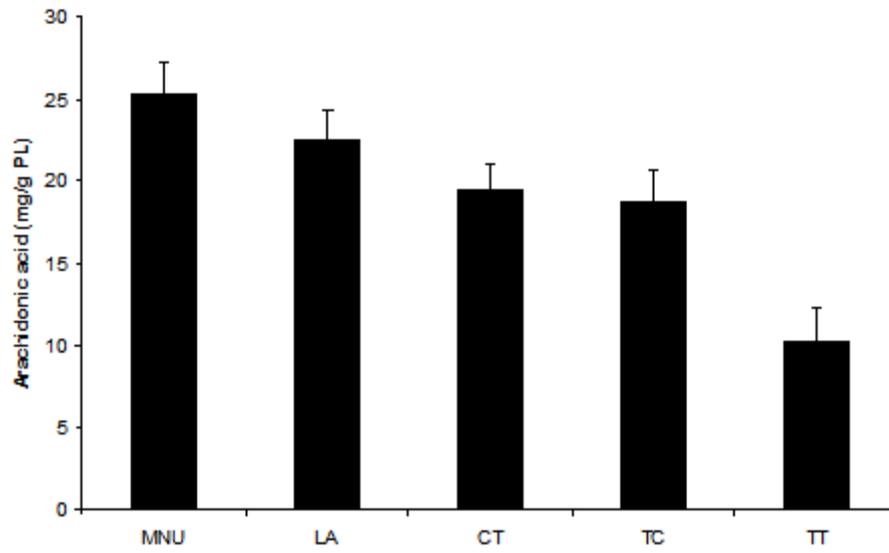
**Figure 6:** Effects of t,t CLAs on the expression of PPAR-gamma proteins in MNU-induced rat mammary tumorigenesis was determined by western blotting analysis. It was observed that t,t CLAs reduced the expression of PPAR-gamma expression level reference with linoleic acid, c9,t11 CLA and t10,c12 CLA. The MNU, LA, CT, TC, and TT represent control, linoleic acid, c9, t11 CLA, t10, c12 CLA, and t,t CLA, respectively. The band intensities relative to the control were quantified. Values are means  $\pm$  SD (n = 3). Means with different small letters are significantly different at  $p < 0.05$



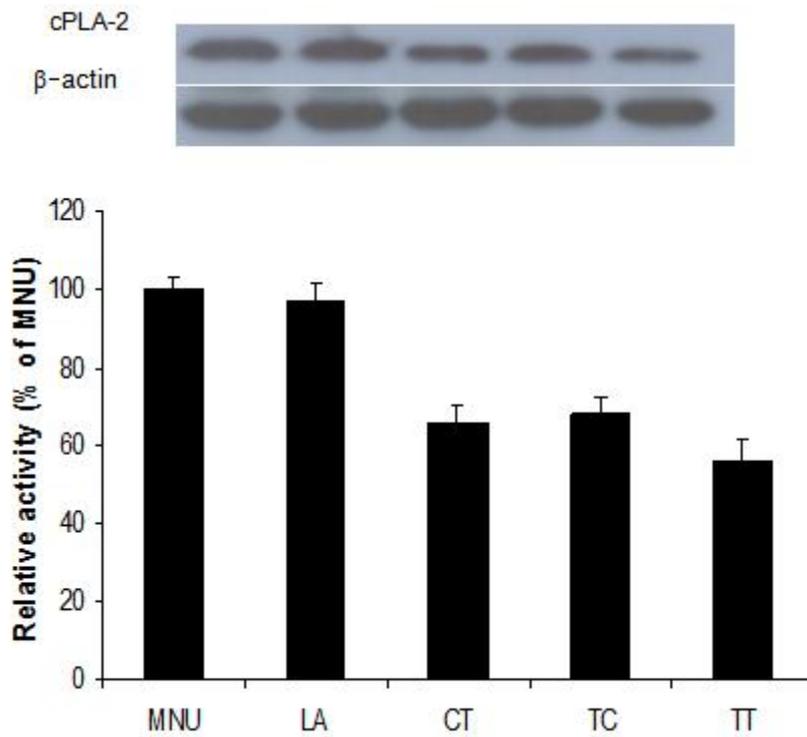
**Figure-1**



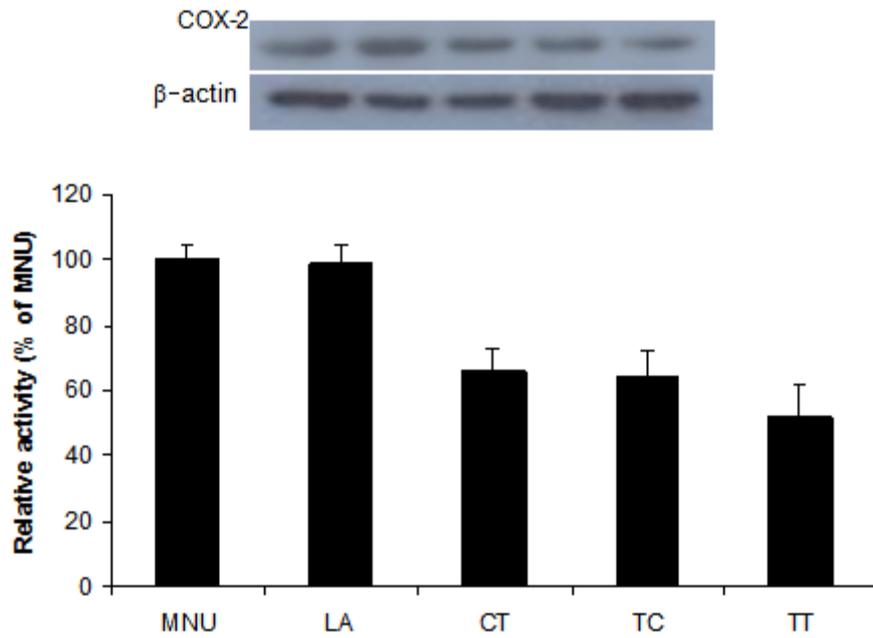
**Figure 2**



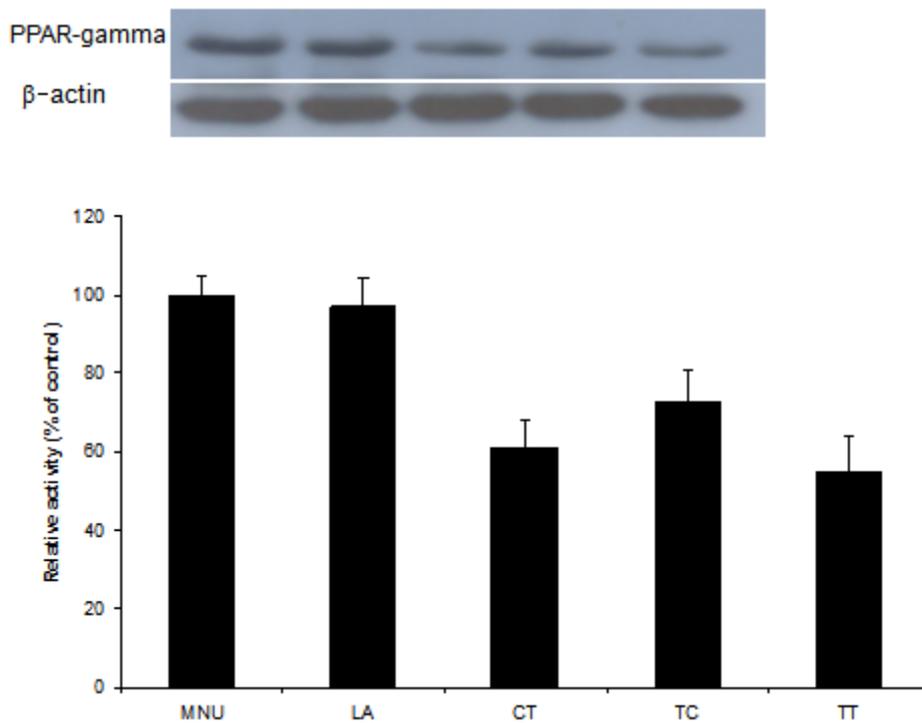
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**