

Study the Effect of Indomethacin Administration on Breast Tumor Growth and Inos Gene Expression in Tumor-Bearing Mice

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ABSTRACT

Breast cancer is a public health concern among the women. iNOS is stated by the effect of various inflammatory factors and is thus called inducible NOS. Investigating iNOS expression is a potent tool for understanding effective molecular parameters in tissue and cellular responses to external factors. In this research study, iNOS expression in mice with breast cancer was investigated and the effects of various doses of indomethacin on the iNOS gene expression in breast tumors was evaluated.

4T1 cells were grown in RPMI 1640 Medium. 200 µl of cell suspension (10^7 cells) were injected into the mice right flank, subcutaneously. 35 Balb/C albino mice divided into five groups each group consist of 7 mice. First group was healthy control group, second group was tumor control, and the 3rd, 4th and 5th groups were treated by 25µg, 50µg and 100µg of indomethacin, respectively. The rate of breast tumor growth was measured in treated groups for two months. The levels of iNOS gene expression was determined using real time RT-PCR technique.

Our results demonstrated, iNOS expression in tumor tissue increased with the growth of breast cancer cells. Furthermore, Administration of 25µg and 100µg indomethacin neither had significant effect on breast tumor growth nor effect on iNOS gene expression. While 50µg indomethacin decreased the tumor growth ($P>0.05$) and decreased iNOS gene expression.

Further studies such as evaluating the effects of indomethacin on the other anti-tumor immunosuppressing factor and additional tests western blot, flow cytometry and immunohistochemistry recommended in order to obtain more practical findings.

Key words: Indomethacin, gene expression, iNOS, breast tumor.

Introduction

Cancers mortality augmented during the years (Gürsoy *et al.*, 2009), now it is the second cause of death in developed country and in Iran is the third cause of death after cardiovascular diseases and accidents In Iran, breast cancer is the outstanding type of

cancer accounting about 24.6% of different types of cancers for females (Jazayeri *et al.*, 2015).

In different kinds of cancers such as breast cancer, colon cancer and melanoma, over expression of iNOS was observed (Cianchi *et al.*, 2003; Granados-Principal *et al.*, 2015; Grimm *et al.*, 2008) while in esophageal cancer the results illustrated that there was no significant difference between iNOS expression in the esophageal cancer group and control groups ($p > 0.05$); however, there was an escalation in iNOS expression in the case group. Interestingly, there was a significant difference between iNOS expression in males and females in the two groups of healthy subjects and patients, and it was higher in women than in men (Barani *et al.*, 2016).

Inducible nitric oxide synthase (iNOS) is a pro-inflammatory enzyme which has effects on chronic inflammation and wound healing and it could synthesize Nitric Oxide (NO) (Nathan *et al.*, 1994).

NO is a tiny bioactive molecule with pleiotropic effects and has variety of effects on cancer cells and tumors biology such as dose and temporal dependent via the capability to inhibit or motivate cell proliferation, migration and apoptosis. Nitric oxide also has concentration-dependent pro- and anti-tumor effects (Ambs *et al.*, 2011; Burke *et al.*, 2013; Pervin *et al.*, 2007). NO is released by different NOS isoforms such as neuronal (nNOS/NOS1), inducible (iNOS/NOS2) and endothelial (eNOS/NOS3) (Burke *et al.*, 2013) all of which are encoded by separate genes and, therefore, differently regulated. Unlike endothelial NOS and neuronal NOS, iNOS is not expressed constitutively, but is expressed in most cell types given the appropriate stimulatory conditions, which include infection, cytokines, mechanical injury, and hypoxia (Xie *et al.*, 1994).

Generally, neuronal NOS and endothelial-cell NOS produce NO in low concentrations, and they can be induced in neurons and Endocrine cells. Their activity depends on high levels of cytoplasmic calcium/calmodulin. However, iNOS can be produced by neutrophils, macrophages, endothelial cells and other cell type (Nathan *et al.*, 1994).

Research studies showed that stem cell-like tumor properties induced by NO in breast cancer (Ambs *et al.*, 2011). Pro-inflammatory signaling pathways are generally up-regulated in breast cancer, nitric oxide synthase-2 (NOS-2) showed as independent predictors of diseases result (Basudhar *et al.*, 2017). Nitric oxide synthase-2 (NOS2) is an inflammation-associated enzyme which has oncogenic function in breast cancer (Basudhar *et al.*, 2017).

The anti-inflammatory drugs have a great potential on increasing the effectiveness of cancer immunotherapy drugs (Hussain *et al.*, 2012). Indomethacin is none steroidal anti-inflammatory drugs (NSAIDs) which has harmful effect on gastrointestinal (GI) mucosa (Carrasco-Pozo *et al.*, 2016). Also, Indomethacin has effect on some cancers such as colorectal cancer (Cianchi *et al.*, 2003), skin cancer, head and neck carcinoma, melanoma and B cell lymphoma (Grimm *et al.*, 2008), small cell lung cancer cells and pancreas cancer in vitro (Lange *et al.*, 2011; Okayama *et al.*, 2013). In various studies, indomethacin had anti-cancer activity in inhibition of cell growth, induction of apoptosis and suppression of tumor invasion (Okayama *et al.*, 2013).

Although many studies have been done on different kinds of cancers, little attention has been given, up to now, to the study on the effect of indomethacin on iNOS gene expression. In this paper we aimed to: 1) investigate the effect of various doses of indomethacin on

iNOS gene expression and 2) assess the tumor growth rate in breast tumors in different doses of indomethacin.

Materials and method

Breast cancer cell line 4T1 was purchased from Pasteur institute Tehran Iran. Breast cancer cells line were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (Invitrogen, UK) which is containing 10% fetal bovine serum (FBS), L-Glutamine, 100 units/mL penicillin and 200 µg/mL streptomycin. Cells were maintained in a humidified atmosphere with 5% CO₂ at 37 °C, with exchange of medium every 48 h.

35 Female Balb/c albino mice (6–7 weeks of age, 14-15 g in weight) were purchased from Pasteur institute Tehran Iran. 200µl suspension contain 10⁷ cells, mycoplasma free were induced in mice's right flank subcutaneously.

Three weeks after implantation of tumor cells mice with 80-100 mm³ tumor volume were divided into 5 groups each group contain 7mice. The mice were housed in cages maintained in a regulated environment (12-h: 12-h light/dark cycle) and were supplied with water and food ad libitum. First group was healthy control (to 7 mice), second group was tumor control (to 7 mice) and 3rd, 4th and 5th were tumor groups which received 25, 50 and 100 µg indomethacin, respectively. First the amount of drug were dissolved into distilled water and fed the mice orally for two months. For both healthy and tumor control groups plovise were administrated. The size of tumor were measured with digital caliper $\frac{1}{2}$ (length× width²) in 10, 17, 23, 30 and 37 day after treatment (Figure 1). All institutional guidelines for animal welfare and experimental conduct were followed. All efforts were made to minimize animal suffering. Briefly, the mice with physical approach, cervical dislocation were euthanized. 100 mg of tumor was collected and stored in RNA Later (Behnogen). The biopsy was lysis with Guanidine thiosulfate lysis buffer (KIAzol®, USA).

iNOS mRNA expression was detected in the treated mice and compared with the control mice. cDNA synthesis was performed. The concentration and purity of standard cDNA was determined by Thermo Scientific™ NanoDrop™ 2000/2000c.

The primer sequences used for iNOS were forward, 5'-GGAGCGAGTTGTGGATTG-3' and reverse, 5'-CCAGGAAGTAGGTGAGGG-3'. The primer sequences used for β-actin were forward, 5'-GAAGATCAAGATCATTTGCTCC-3' and reverse, 5'-CTAAGTCATAGTCCGCCTAG-3'. For PCR, a two-step RT-PCR kit (Sinagene) was used. Preparation steps of major PCR solution were performed on ice and all tubes were placed on ice. The PCR reactions for iNOS were initiated with a denaturing step at 95°C for 3 min, followed by 40 cycles at 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 20 s PCR was performed by a thermocycler machine (Mastercycler gradient, Eppendorf).

Real-time PCR reaction was performed for analyzing iNOS gene expression. β-actin was used as an internal control gene. SYBR green I was used for performing real-time PCR reaction. The composition and amount of reaction components of real-time PCR were observed. After placing samples in the system for real-time PCR reaction, the system's software was programmed. A fluorescent reporter was used for this method. These reporters are designed in a manner that if they have DNA replication, they should produce light, so increasing recorded light intensity in the device is directly proportional to the amount of obtained product. By continuing PCR, the fluorescent intensity will increase. The first cycle in which the fluorescent is greater than the baseline threshold is called threshold cycle (CT), in other words, in the initial phase of progressive steps, the amount of

fluorescent increases to reach a threshold, and it is higher than the background level with a known amount, and this cycle is called CT. The CT amount was recorded based on drawing a threshold for all samples. For each sample, three replications were performed and the average CT was calculated for all samples. Information processing in real time PCR is important. The investigate ways were $\Delta\Delta CT$ method. To reduce errors and correct variations in primer materials in RT-PCR, the approved method is simultaneous RNA amplification, which is used as an internal standard. In this study, data were tested by using the t test in IBM SPSS statistics 22 software significance level set at 0.05.

Results

The CTs of the samples were calculated based on the proliferation diagrams after performing reaction on all samples of any concentration using β -actin protein and their curvature drawn (based on the CT on the y-axis and the log of the copy amount on the x-axis). The efficiency of the reaction for β -actin was 1.07 and R^2 was 98% after drawing the standard curvature.

The same steps performed for the β -actin standard curvature were performed for the iNOS standard curvature as well. The efficiency of the reaction for iNOS was 90% and R^2 was 97% after drawing the standard curvature.

The β -actin proliferation progression in every cycle was observed. As well as, the progression of iNOS proliferation curvature in every cycle was observed.

One of the most important advantages of real-time PCR is fusion curvature drawing by which we determined the diversity of products in the PCR reaction. In order to draw the curvature the instrument changes the samples temperature in specified durations, then the rate of change is shown on the y-axis and the instrument temperature is shown on the x-axis. In order to draw the curvature, samples temperature increase and the light which was emitted from the samples were measured. There was a sudden drop of florescent light intensity at about 75 °C was observed.

The fusion curvature was evaluated in order to observe whether or not PCR worked properly. In this curvature the temperature of the samples increases gradually and the emitted light was measured. The PCR product of iNOS begins to open in becoming single-strand following increasing the temperature of double-strand DNA and dye molecule separate from them. The intensity of fluorescence decreases following increasing the temperature until the temperature reaches around 74 °C, which is the temperature of the PCR reaction product, then the intensity of fluorescence decreases and breaking will appear in the fusion curvature (Figure 4, 5).

Statistical results obtained from the analysis demonstrated, that iNOS gene expression increased in tumor tissue while breast cancer cells were growing.

To ensure amplification of the gene, the samples resulted from PCR amplification, four amplified samples of each gene were randomly chosen and were put on 2% agarose gel the results obtained from agarose gel image confirmed the correct amplification of iNOS and β -actin (Figure 3).

In this study, the administration of two doses of indomethacin, 25 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$ neither had any significant effect on tumor growth nor effect on iNOS gene expression.

All mice which were treated with 100 $\mu\text{g}/\text{mL}$ of indomethacin were died within 14 days after treatment.

Interestingly, in one hand, 50 µg/mL of indomethacin considerably reduced the iNOS gene expression in treated group in comparison with control group (Figure 2). In the other hand our results demonstrated that, 50 µg/mL of indomethacin decreased tumor growth but this reduction was not significant (Figure 1).

Also the group which was treated with 50 µg/mL indomethacin had longer survival time (about 60 percent of the mice were alive 2 weeks longer).

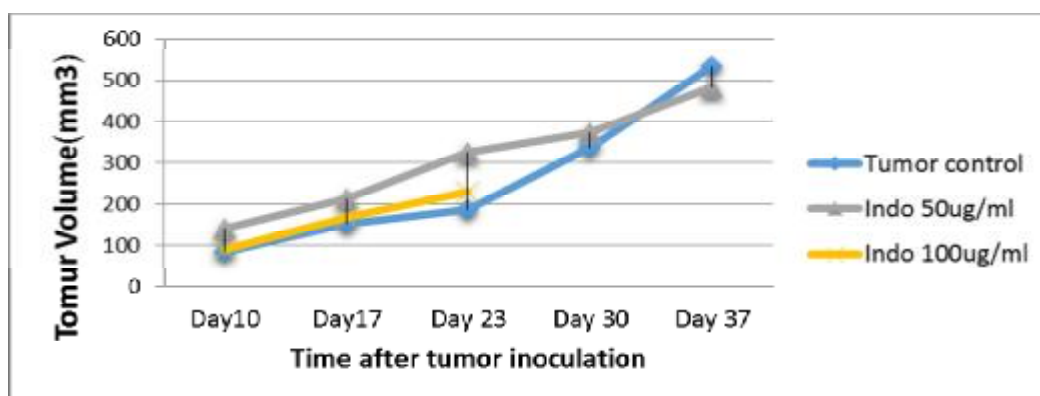


Figure 1. Tumor growth after indomethacin administration in drinking water

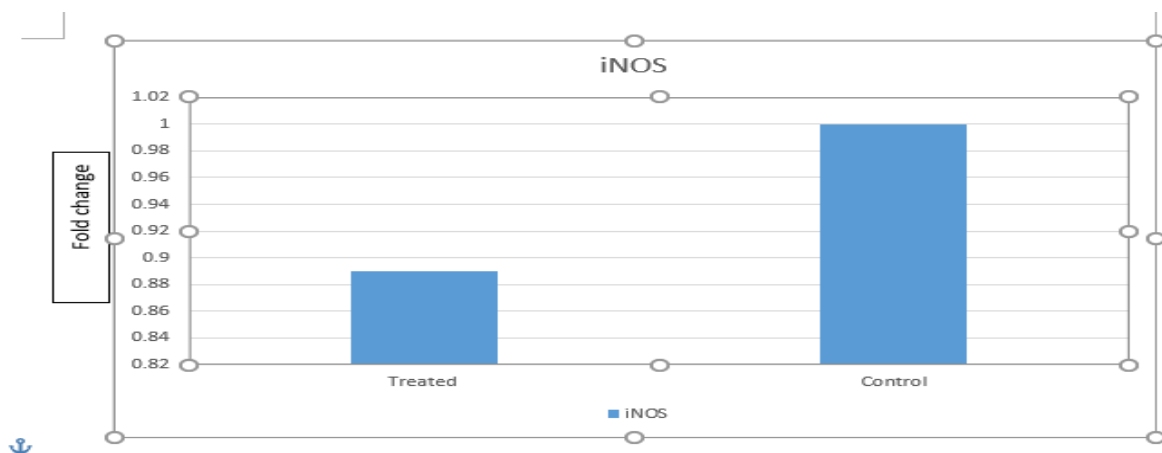


Figure 2. 50 µg/mL indomethacin reduced the expression of the iNOS gene in compared with the control group

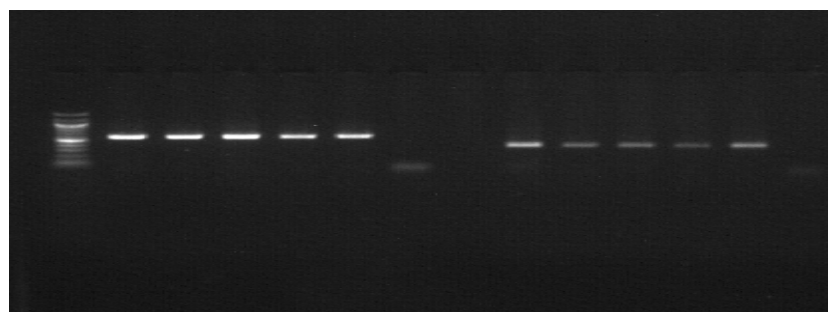


Figure 3. PCR amplification agarose gel image

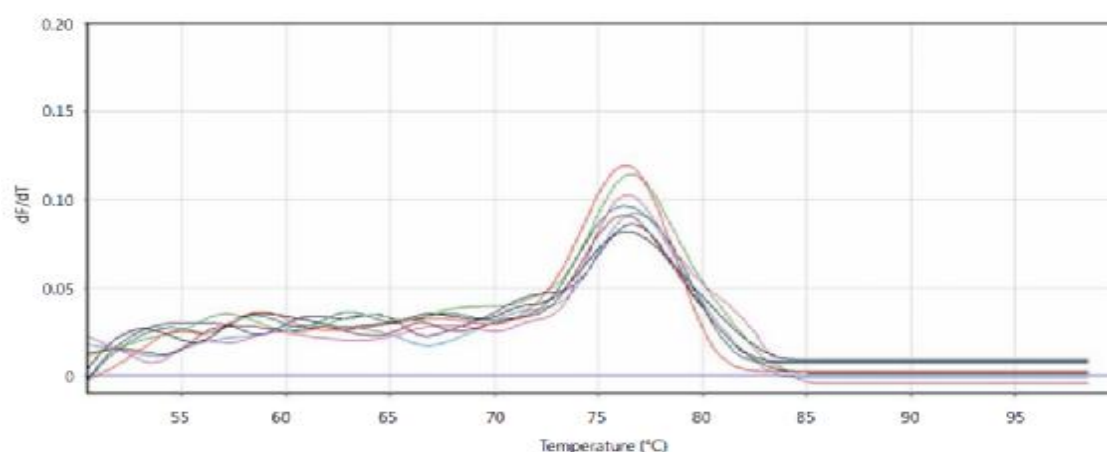


Figure 4. iNOS fusion curvature. 77 °C was the temperature of iNOS product

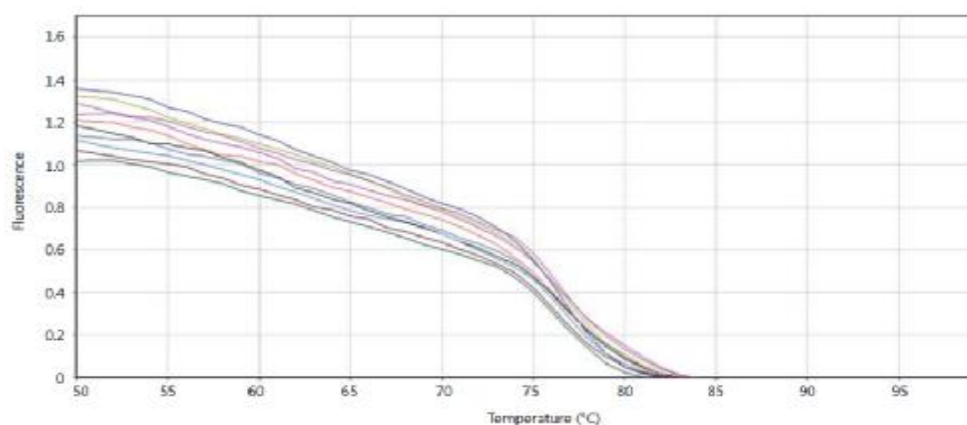


Figure 5. The changes of fluorescence in terms of temperature for iNOS gene

Discussion

Breast cancer is the predominant cause of mortality among women worldwide (Vahora *et al.*, 2016). In Iran, it is the first leading cause of cancer-related deaths among women (Jazayeri *et al.*, 2015). Despite numerous advances, treatment resistance and metastasis are the main causes of death in patients with breast cancer (Granados-Principal *et al.*, 2015). Nitric oxide (NO) is a bioactive molecule that exhibits pleiotropic effects within cancer cells and tumors, with concentration-dependent pro- and anti-tumor effects. In mammals, NO is synthesized by three different nitric oxide synthase (NOS) isoforms: neuronal (nNOS/NOS1), inducible (iNOS/ NOS2), and endothelial (eNOS/NOS3) (Burke *et al.*, 2013). Increased iNOS expression has been found in breast cancer (Bulut *et al.*, 2005) and other different cancers such as lung (Okayama *et al.*, 2013), colon (Ambs *et al.*, 2011), melanoma (Massi *et al.*, 2001), and glioblastoma (Eyler *et al.*, 2013).

The results of our study showed that iNOS expression increased in breast cancer cells (Figure 3 and Figure 4).

In many tumors, iNOS expression is high, however, the role of iNOS during tumor development is very complex and quite perplexing, with both promoting and inhibiting actions having been described (Vannini *et al.*, 2013). Overexpression of iNOS and

concomitant changes on cancer cell kinetics were demonstrated to be anti-cancer in potential according to several in vitro and in vivo studies. In animal models of fibro sarcoma tumor progression slowed down upon expression of iNOS via gene transfer demonstrating potential of tumor growth inhibition by iNOS-derived NO. Overexpression by means of other delivery forms of iNOS also produced anti-tumor results (Vannini *et al.*, 2013).

Retroviral delivery of iNOS in mice produced slowly progressing tumors and fewer lung metastases than control mice transfected with iNOS-negative retrovirus (Juang *et al.*, 1997). Increased iNOS activity has been positively correlated with the degree of malignancy in gynecological tumors (Thomsen *et al.*, 1995), gastric cancer, squamous cell carcinoma, hepatocellular carcinoma, melanoma and leukemia (Bonavida, 2010), while high iNOS expression is associated with favorable prognoses in ovarian (Anttila *et al.*, 2007) and lung cancers (Puhakka *et al.*, 2003). Osteosarcoma of the jaw in patients was associated with over expression of iNOS as deduced by immunohistochemical analysis (Chen *et al.*, 2007). Similarly, oral carcinomas and oral hard tissue sarcoma in patients were also associated with iNOS expression. Interestingly, soft tissue oral sarcomas from this study were negative for iNOS expression (Augustine *et al.*, 2015) and iNOS expression in carcinomas was higher than in sarcomas. In women with estrogen receptor (ER)-negative breast tumors, iNOS expression is an independent marker for prognosis. iNOS positivity related with angiogenesis of tumors (Glynn *et al.*, 2010). In other clinical studies of gastric cancer, iNOS correlated with tumor progression and has potential to be of prognostic value (Li *et al.*, 2005). Low levels of expression of iNOS produced tumors in the pancreas and led to liver metastasis and ascites in mice models while higher levels of iNOS expression did not lead to metastasis. When iNOS expression was inhibited, it promoted distant liver metastasis in these mice (Wang *et al.*, 2003). While some research studies have demonstrated a correlation between high iNOS expression, aggressiveness, and poor prognosis in patients with breast cancer (Bulut *et al.*, 2005).

Our present results demonstrate that Indomethacin may inhibit iNOS expression depending on endogenous levels, as the administration of 50 µg/mL dose of indomethacin remarkably reduced the iNOS gene expression in treated group in comparison with control. Also, it was observed that 50 µg/mL dose of indomethacin was markedly increased survival time of treated group in comparison with control group and it was associated with poor tumor growth of treated group with 50 µg/mL dose of indomethacin (Figure 1). The inhibitor role of Indomethacin on iNOS expression has not been previously studied. Indomethacin is a non-steroidal anti-inflammatory drug (Glynn *et al.*, 2010). The usage of non-steroidal anti-inflammatory drugs reduced the risk of breast cancer (Yiannakopoulou, 2015). The administration of this drug resulted in the inhibition of tumor growth or tumor cell proliferation (Fulton, 1984). Some researchers demonstrated Indomethacin has anti-tumor effect on some cancers such as pancreatic and lung cells (Lange *et al.*, 2011), and it increased the apoptosis in colon cancers cell (Jazayeri *et al.*, 2015). (Narisawa *et al.*, 1981) reported that at autopsy, after the 15-week treatment with indomethacin, a prostaglandin synthesis inhibitor, the colonic tumor incidence was significantly lower in treated rats than in untreated control rats. In another study, treatment of mice with indomethacin significantly inhibits tumor development (Shacter *et al.*, 1992).

In various studies, indomethacin anti-cancer activity in inhibition of cell growth, induction of apoptosis and suppression of tumor invasion by regulating the expression of adhesion molecules have been attributed. In nude mouse model of human medullary thyroid cancer

which treated by indomethacin the tumor volume (dimension) decreased (Carrasco-Pozo *et al.*, 2016).

Conclusion

Finally, our results clearly demonstrated that iNOS gene expression was remarkably expressed in breast tumor tissue ($p < 0.05$). 50 $\mu\text{g/mL}$ indomethacin in one hand decreased the iNOS gene expression and on the other hand reduced tumor growth. However there was no significant difference in tumor growth between the studied groups ($p > 0.05$). Further studies and research should be done such as evaluating the effects of indomethacin on the other anti-tumor immunosuppressing factor. Performing some additional tests like western blot, flow cytometry and immunohistochemistry need to complete our results.

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