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ASPISOL INHIBITS TUMOR GROWTH AND INDUCES APOPTOSIS IN BREAST CANCER

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Nonsteroidal anti-inflammatory drugs inhibit cell proliferation and induce apoptosis in various cancer cell lines, which is considered to be an important mechanism for their anti-tumor activity and cancer prevention. However, the molecular mechanisms through which these compounds induce apoptosis are not well understood. *Aim:* to determine the effects of nonselective cyclooxygenase-2 (COX-2) inhibitor, aspisol on breast cancer cells *in vitro* and *in vivo*. *Methods:* The cytotoxic activity of aspisol was evaluated by MTT assay. The apoptosis index of cells was measured by flow cytometry. Immunohistochemical staining was used to detect expressions of COX-2 and caspase-3 in MDA-MB-231 cells. The expression of bcl-2 and bax was analyzed by Western blot analysis. The content of prostaglandin E_2 (PGE_2) in MDA-MB-231 cells was estimated by ELISA. *In vivo* apoptosis of the tumor cells was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). *Results:* Our results showed that aspisol reduced viability of MDA-MB-231 cells in time- and dose- dependent fashions and induced apoptosis by increase of caspase-3 and bax expressions while decrease of COX-2 and bcl-2 expression *in vitro*. In addition, exposure to aspisol decreased the basal release of PGE_2. *In vivo*, aspisol also inhibited the proliferation of breast cancer cells and induced their apoptosis. *Conclusions:* Our *in vitro* and *in vivo* data indicated that the antitumor effects of aspisol on breast cancer cells was probably mediated by the induction of apoptosis, and it could be linked to the downregulation of the COX-2 or bcl-2 expression and up-regulation of caspase-3 or bax expression.
Key Words: **aspisol, NSAIDs, apoptosis, COX-2, breast cancer cells.**

Breast cancer is the second most common cause of cancer death in women [1]. The incidence of breast cancer is increasing but current therapy is unable to achieve clinical responses in patients with this highly invasive metastatic disease. There is a consequent need for more effective approaches to prevention and treatment of breast cancer. Although many cancers initially respond to chemotherapy, resistance often develops. Because many breast cancer patients treated by standard schemes suffer from undesirable side effects [2], studies of new approaches of breast cancer treatment should be continued.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are well known to inhibit cyclooxygenase (COX) activity, the key enzyme in prostaglandin biosynthesis. However, several clinical observations, epidemiological and experimental studies showed that NSAIDs could be promising anti-cancer agents. COX-2 overexpression was found in breast cancer tissues and it was associated with poorer prognosis [3]. Epidemiological studies as well as early clinical trials suggest that administration of either dual COX-1/COX-2 or selective COX-2 inhibitors may reduce the risk of cancer development [4]. Preclinical studies also indicated that the inhibition of COX is useful in animal models of chemoprevention [5]. Inhibition of COX-2 can decrease breast cancer cell motility, invasion and matrix metalloproteinase expression [6]. Aspirin has been shown to be associated with lower risks of cancer incidence and mortality [7]. It was reported recently that the use of NSAIDs for 5–9 years for more than 10 years reduced the incidence of breast cancer by 21% and 28%, respectively [8]. Other studies showed that aspirin and non-aspirin-NSAIDs contributed to breast cancer prevention in the general population [9, 10], and NSAIDs induced apoptosis of tumor cells [11–13]. But the molecular mechanism of NSAIDs-mediated apoptosis is still unclear. Preclinical trials are needed to determine whether NSAIDs could be used for prevention and/or treatment of breast cancer. In spite of the established role of COX-2 and NSAIDs in human cancer, little is known about the effect and mechanism of NSAIDs in the growth control of breast cancer cells. In the present investigation, we demonstrated that aspisol reduced MDA-MB-231 cells viability, induced their apoptosis by increasing the expression of caspase-3 and bax, and decreased the expression of COX-2 and bcl-2. Also, we demonstrated that aspisol inhibited tumor growth of and induced tumor cells apoptosis in C3H mice model.

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Abbreviations used: COX-2 – cyclooxygenase-2; FCM – flow cytometry; PGE_2 – prostaglandin E_2; NSAIDs – nonsteroidal anti-inflammatory drugs; TUNEL – terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

MATERIALS AND METHODS

Cell Culture and Drug Treatment. The human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in Dulbecco's modified eagle medium (DMEM) (GIBCO-BRL, Rockville, USA) supplemented with 10% fetal bovine serum (FBS), 100 U penicillin, 0.1 μg streptomycin and 2 mmol/L L-glutamine at 37 °C, with 5% CO_2. The cells were plated in the regular medium for 24 h, which was then replaced by either control fresh FBS-free medium or the medium containing 1, 5, or 10 mM of aspisol (Fengyaun, Anhui, China). Drugs were dissolved directly in DMEM.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. MTT assay was used to measure cell viability. Briefly, 2×10^4 MDA-MB-231 cells were seeded in 96-well plates in 180 μl of medium, and incubated in medium containing different concentrations of aspisol (1–10 mM) for 24 h, 48 h and 72 h. 20 μl of MTT

(5 mg/ml in PBS) (Sigma, USA) was added to each well, and the cells were incubated for an additional 4 h. Blue formazans were released from the cells by adding 150 µl DMSO with gentle shaking at 37 °C, and absorbance was measured at 570 nm using a microplate reader (Bio-Tek Instruments, Richmond, USA). Percent of viabile cells was defined as the relative absorbance of treated cells *vs* untreated control cells.

Western blot analysis. Following aspisol treatments, MDA-MB-231 cells were washed twice with ice-cold PBS and harvested in sample buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 µg/ml aprotinin. Soluble extracts were prepared by centrifugation at 12 000 rpm for 30 min at 4 °C. Protein concentrations were determined by the Bradford assay. Equivalent amounts of protein (40 µg) for each sample were resolved in 12% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes and blocked by 5% nonfat milk for 1h. Antibodies used for Western blot analysis included rabbit anti-bcl-2 antibody, rabbit anti-bax antibody (Cell Signaling, USA) and mouse anti-alpha-tubulin antibody (Sigma, USA), horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, USA).

Flow cytometry analysis. 24 h after the treatment of cells with aspisol poptosis was determined by staining the cells with annexin V and propidium iodide (PI) using apoptosis kit from BD Pharmingen (San Diego, USA). The percentage of stained cells in each quadrant was quantified using Winmdi 2.9 software.

Immunohistochemistry. To evaluate whether aspisol treatment could modify caspase-3 and COX-2 expression of, we detected caspases-3 and COX-2 expression treated and untreated cells by immunohistochemical staining. 2×10^5 cells were seeded in triplicates in 6-well plates on coverslips, and grown for 24 h. Medium was then replaced with media containing aspisol (1–10 mM) and cells were grown for the additional 12 h. The cells on coverslips were fixed by 4% paraformaldehyde solution, and then were dehydrated in alcohol. Endogenous peroxidase was blocked by 3% H_2O_2 in methanol and avidin/biotin (Vector Laboratories, Burlingame, CA). The coverslips were incubated overnight at 4 °C with 1 : 500 dilutied rabbit anti-caspase-3 or rabbit anti-COX-2 (Santa Cruz, USA) specific antibody. All appropriate controls were made.Immunoreactive complexes were detected using tyramide signal amplification (TSA-indirect) and visualized with the peroxidase substrate, AEC. Coverslips were counter stained with hematoxylin.

Detection of PGE_2 level in culture media. MDA-MB-231 cells were treated with increasing concentrations of aspisol (1–10 mM). Level of PGE_2 released to culture media was measured using a PGE_2 enzyme immunoassay kit (Cayman Chemical, USA). Medium was sampled, centrifuged to remove floating cells and immediately frozen at –70 °C before analysis. The PGE_2 assay was performed according to the manufacturer's instructions. The results were expressed relatively to the control. Data presented are the results of at least three independent experiments done in triplicates.

Tumor proliferation in C3H mice. Female C3H mice were obtained from the Animal Production Area of China Medical University. The mice were maintained under specific-pathogen-free conditions. We used 6-week-old mice weighing 18 to 22 g, acclimatized for one week before starting the experiments. C3H mice spontaneous mammary adenocarcinoma cells were injected subcutaneously into forelimb axillas of C3H mice (1×10^7 cells per mouse). In 24 h mice were treated with vehicle (normal sodium), 5-fluorouracil (5-FU, 10 mg/kg), aspisol (300 mg/kg/day) for 4 weeks. Each group comprised of 10 animals. The tumor volume (TV) was assessed every 3 days by using a calliper measuring of the two major diameters by the formula $TV = d1 \cdot d2^2 / 2$. After administration of the last dose (24 h) mice were killed, the tumors were excised, fixed and sliced into 2-mm-thick sections for analysis tumor apoptosis with Terminal dUTP nick-end-labeling assay (TUNEL) assay. The TUNEL assay was performed as directed by the manufacturer. Endogenous peroxidase was blocked in scetions, and they were treated with 0.25% Triton X-100 in PBS at 50 °C for 20 min, and incubated with terminal deoxytransferase enzyme with biotin dUTP and cobalt ions for 90 min at 37 °C. Anti-BrdUrd and TUNEL-labeled sections were visualized with streptavidin peroxidase and diaminobenzidine (Dako Corp, Carpinteria, CA), followed by hematoxylin staining. Apoptotic nuclei were stained dark brown, and normal cell nuclei were blue. The animal experiments were approved by the local Ethics Committee for Animal Research.

Statistical analysis. All data were expressed as mean ± SD and analyzed by one-way of variance (ANOVA) or Student's t-test using SPSS software (version 11.0 for Windows). Significance was accepted at $P < 0.05$.

RESULTS

Aspisol inhibited MDA-MB-231 cell viability. Our results suggested that treatment with aspisol reduced cell viability in dose-dependent manner. Lower concentrations of aspisol (5 and 10 mM) significantly reduced MDA-MB-231 cells viability in 72 h (Fig. 1).

Aspisol induced dose-dependent apoptosis in MDA-MB-231 cells. Following 24 h of drug treatment, induction of apoptosis was observed in the MDA-MB-231 cells in a dose-dependent manner (Fig. 2). Aspisol at 5 and 10 mM caused the increase in apoptotic cells.

COX-2 and caspase-3 expression in MDA-MB-231 cells. To determine whether the effect of aspisol was associated with COX-2 caspase-3 expression MDA-MB-231 cells, immunohistochemical analysis was performed. It was found that COX-2 was consistently expressed by MDA-MB-213 cells, and there was significant down-regulation of COX-2 expression upon aspisol treatment (Fig. 3). Treatment of MDA-MB-231 cells

with 5 and 10 mM aspisol for 12 h caused significant increase in the caspases-3 expression (Fig. 4). Obtained results suggested that aspisol-induced apoptosis in MDA-MB-231 cells correlated with COX-2 down-regulation and caspase-3 in these cells.

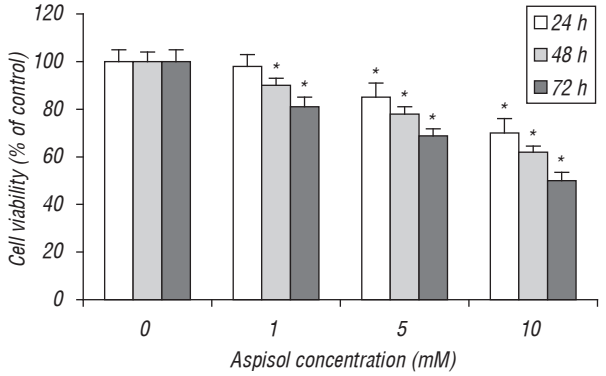

Fig. 1. Aspisol inhibits MDA-MB-231 cell viability. MDA-MB-231 cells were treated for 24, 48, or 72 h with 0, 1, 5, or 10 mM aspisol. Cells viability was determined by MTT analysis. Aspisol significantly inhibits the viability of MDA-MB-231 cells in a dose-dependent manner. There is a significant difference between control and aspisol treatment (*$P < 0.05$). Experiments were repeated three times, with similar results

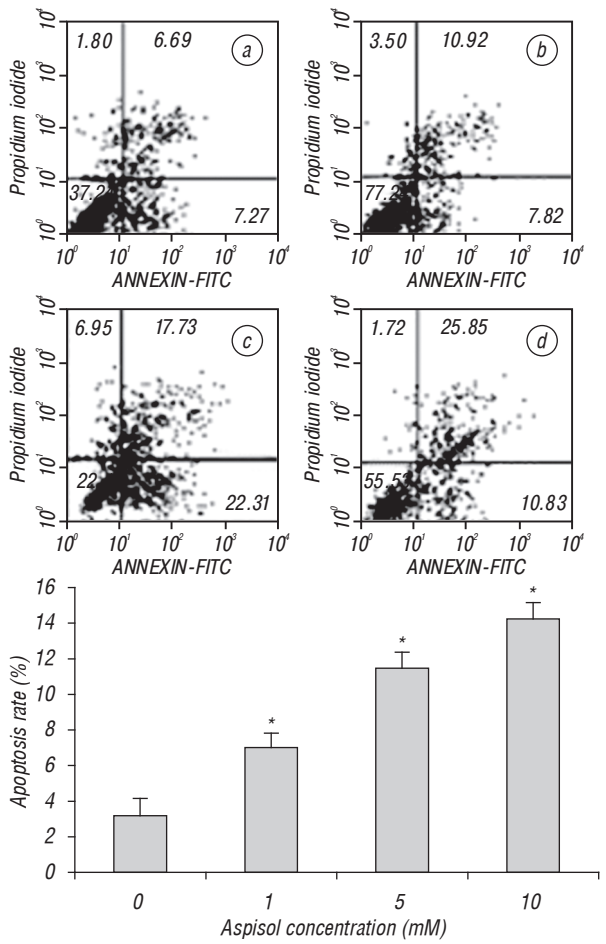

Fig. 2. Aspisol induces apoptosis in MDA-MB-231 cells. *a*, control; *b*, 1 mM aspisol; *c*, 5 mM aspisol; *d*, 10 mM aspisol. MDA-MB-231 cells were treated for 24 h with 0, 1, 5, or 10 mM aspisol. Apoptosis was then determined by flow cytometry. Data was analyzed by Student's *t*-test. There is a significant difference between control and aspisol treatment (*$P < 0.05$); aspisol increased the number of apoptotic MDA-MB-231 cells in dose-dependent manner. Experiments were repeated three times, with similar results

Aspisol induce the decrease of bcl-2/bax ratio in MDA-MB-231 cells. To determine whether the effect of aspisol is associated with the changes of bcl-2 and bax expression in MDA-MB-231 cells, Western blot analysis was performed. It was shown that exposure to 10 mM aspisol induced the decrease of bcl-2 expression and theincrease of bax expression in treated cells. The bcl-2/bax ratio was decreased to 15.4 ± 5.9% from control (Fig. 5).

Aspisol inhibited COX-2-mediated PGE_2 production by MDA-MB-231 cells. To determine whether COX-2 activity was affected by aspisol treatment, PGE_2 production was mesuared using a PGE_2-specific enzymelinked immunosorbent assay. The results are presented on Fig. 6. Overall, it was shown that aspisol treatment reduced PGE_2 secretion in MDA-MB-231 cells in a concentration-dependent manner (Fig. 6).

Aspisol inhibited tumor growth by inducing cancer cells apoptosis in C3H mice. To assess the relevance of the *in vitro* data, we implanted mammary adenocarcinoma cells subcutaneously into C3H mice. Proliferation of breast cancer xenografts treated with aspisol was significantly reduced (Fig. 7). We observed an increase in TUNEL positive cells in aspisol-treated tumor sections *in situ* as compared with control tumor sections (Fig. 8).

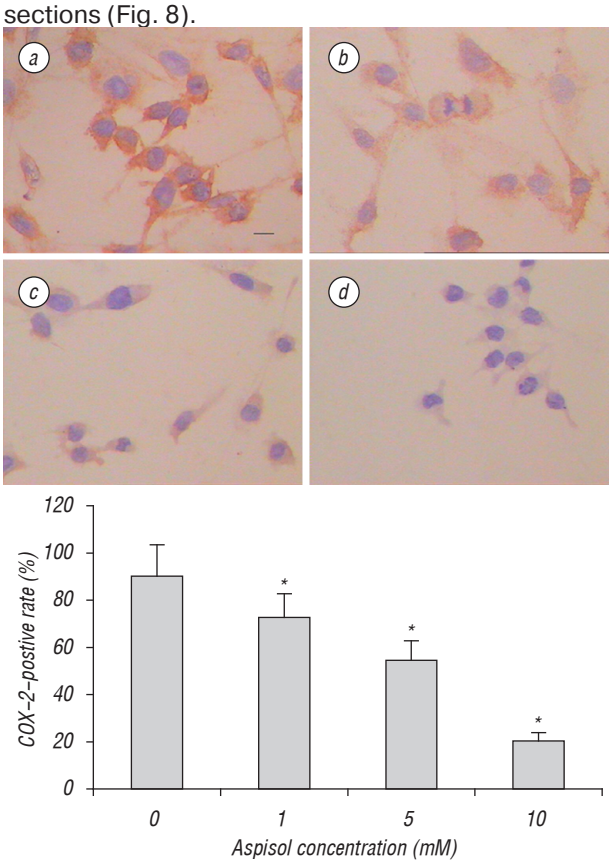

Fig. 3. Aspisol decreases levels of COX-2 in MDA-MB-231 cells. *a*, control; *b*, 1 mM aspisol; *c*, 5 mM aspisol; *d*, 10 mM aspisol. MDA-MB-231 cells were treated for 12 h with 0, 1, 5, or 10 mM aspisol. COX-2 expression was determined by immunohistochemical analysis with specific antibodies. Data was analyzed using one-way ANOVA. *P values represent significant difference between vehicle control and aspisol treatment (*$P < 0.05$). Experiments were repeated three times, with similar results. × 400

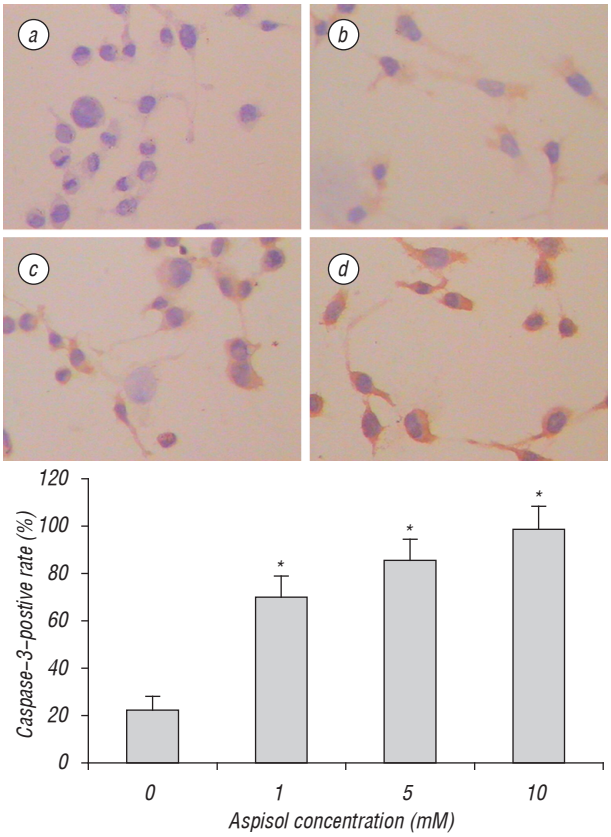

Fig. 4. Aspisol increases levels of caspase-3 in MDA-MB-231 cells. *a*, control; *b*, 1 mM aspisol; *c*, 5 mM aspisol; *d*, 10 mM aspisol. MDA-MB-231 cells were treated for 12 h with 0, 1, 5, or 10 mM aspisol. Caspase-3 expression was determined by immunohistochemical analysis with specific antibodies. Data was analyzed using one-way ANOVA. There is a significant difference between vehicle control and aspisol treatment (*$P < 0.05$). Experiments were repeated three times, with similar results. × 400

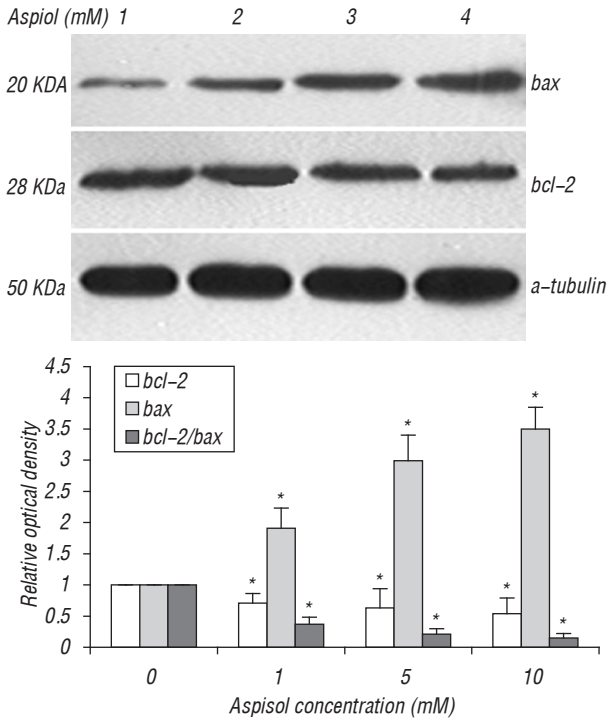

Fig. 5. Aspisol decreases levels of bcl-2 and increases bax level in MDA-MB-231 cells. After treatment with 0, 1, 5, or 10 mM aspisol for 12 h, the protein levels of bcl-2 and bax were examined using Western blotting. Blotting of alpha-tubulin showed equal loading of proteins between each lane. Upper panel shows representative results of three independent experiments. Below panel is bar graph of gray intensities of the immunoreactive bands analyzed by software. The ratio of bcl-2/bax was shown as fold of control. Data were analyzed using one-way ANOVA. There is significant difference between vehicle control and aspisol treatment (*$P < 0.05$). Experiments were repeated three times, with similar results

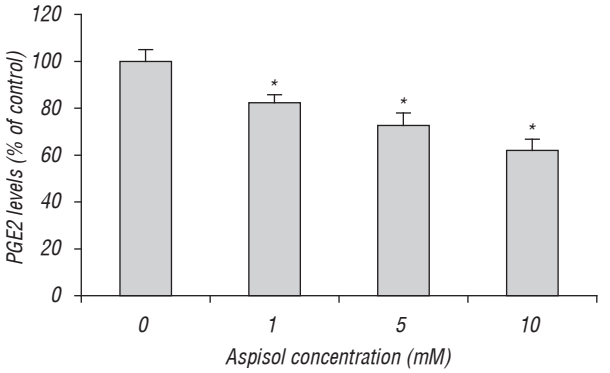

Fig. 6. Inhibition of production of PGE2 by aspisol. MDA-MB-231 cells were cultured for 24 h with the indicated concentrations of aspisol. The amounts of PGE2 in the conditioned medium were determined by ELISA and expressed relatively to the control (*$P < 0.05$). Experiments were repeated three times, with similar results

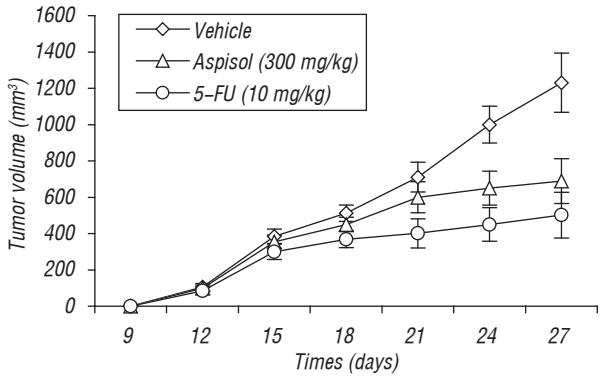

Fig. 7. Treatment with aspisol inhibits the growth of xenografts in C3H mice. Tumors were measured one time/3 days

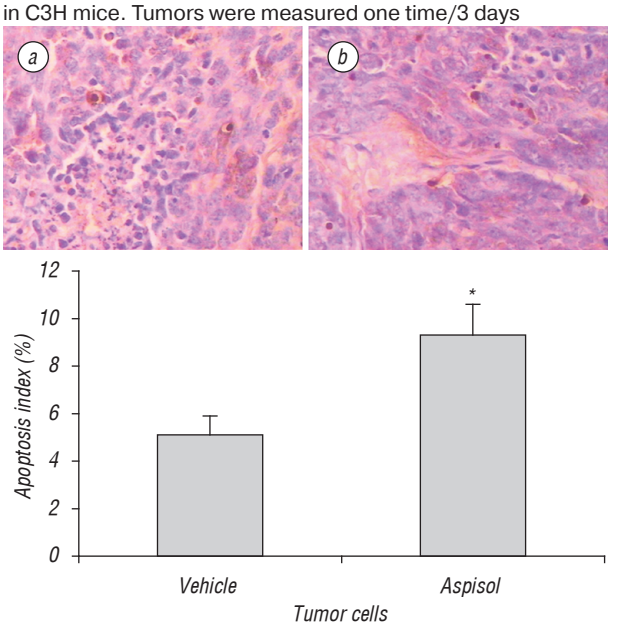

Fig. 8. Effect of aspisol treatment on apoptosis in C3H mice tumor models. TUNEL assay comparison of vehicle- (*a*) and aspisol-treated tumors (*b*) revealed a marked induction of apoptosis in tumor cells in C3H mice models. The apoptosis index of 5% in vehicle-treated tumors increased to 9% in aspisol-treated tumors. × 400

DISCUSSION

COX catalyzes the formation of prostaglandins from arachidonic acid. Overexpression of COX leads to increased amounts of prostanoids in tumors. Prostanoids affect numerous mechanisms that have been implicated in carcinogenesis. PGE_2 can stimulate cell proliferation and motility while inhibiting immune surveillance and apoptosis [14, 15].

NSAIDs are amongst the most commonly used medications worldwide, which can inhibit COX activity. They are considered as effective anti-inflammatory, anti-pyretic and analgesic drugs, and aspirin is also effective in both the primary and secondary prevention of cardiovascular diseases. Aspisol, a new generation of NSAIDs, inhibits both isoforms of COX (COX-1 and COX-2) followed by the decrease of prostanoids level. In this study we examined the effect of aspisol on human breast cancer MDA-MB-231 cells. The obtained results showed that aspisol strongly induced MDA-MB-231 cells apoptosis.

Apoptosis is important in malignancy for two reasons [16]. First, suppression of apoptosis appears to be a critical event in both cancer initiation and progression. Second, most cytotoxic anticancer agents cause tumor regression, at least in part, by inducing apoptosis. Induction of tumor cell apoptosis by NSAIDs is an important mechanism of their antitumor effects [17]. Apoptosis is a tightly regulated process involving changes in the expression or activities of distinct genes [18]. COX inhibitor engages different apoptosis pathways in cancer cells, stimulating death receptor signaling, activating caspases and inducing apoptosis via mitochondrial pathway. Evidence suggests that increase in tumorigenic potential by COX-2 overexpression is associated with resistance to apoptosis. Two distinct isoforms of COX exist, the constitutively expressing COX-1, and the inducible COX-2. COX-1 expresses constitutively in most tissues, whereas the expression of COX-2 is induced by inflammatory factors, hormones and mitogens. COX-1 and COX-2 might all be involved in tumorigenesis. Previous study has shown that COX-1 and COX-2 specific inhibitiors in combined treatment produced the significantly greater inhibition as compared to single agents alone [19]. M.A. Kern *et al.* [20] showed that COX-2 inhibition induced apoptosis in hepatocellular carcinoma cells. Our data revealed that treatment with aspisol caused down-regulation of COX-2 in the cells of breast cancer cell line MDA-MB-231.

Caspases are aspartate-specific cysteine proteases, which cleave their substrates on the carboxyl side of the aspartate residue [21, 22]. Currently at least 14 different caspases are found, of which two-thirds play a role in apoptosis. Caspase-3 is the most widely studied enzyme among other caspases. It was demonstrated to play a key role in both the death receptor pathway, initiated by caspase-8, and the mitochondrial pathway, involving caspase-9. Because caspase-3 is a critical mediator of apoptosis [21] and correlates with apoptosis in breast cancer, it is regarded as a marker for prediction of breast cancer cells' response or resistance to chemotherapeutic agents. We demonstrated that aspisol caused up-regulation of caspase-3 in the MDA-MB-231 cells, suggesting that up-regulation of caspases-3 was involved in aspisol-induced tumor cell apoptosis.

Bcl-2 and bax are other important factors regulating apoptosis. Bcl-2 stabilizes mitochondrial membrane integrity by preventing cytochrome *c* release, and subsequent activation of caspases followed by apoptosis [23, 24]. It has been proposed that the anti-apoptotic bcl-2 protein and the pro-apoptotic bcl-2 family bax proteinare associated with mitochondria-mediated apoptosis through regulation of mitochondrial membrane permeability. The ratio of bcl-2 to bax may ultimately determine the fate of cells [25]. Liu *et al.* [12] confirmed the relationship between COX-2 and bcl-2 family proteins in prostate cancer. Our study showed that aspisol treatment significantly reduced the bcl-2/bax ratio in MDA-MB-231 cells. We found that the levels of COX-2 as well as the bcl-2/bax ratio were decreased in MDA-MB-231 cells upon treatment with aspisol, suggesting that COX-2 and bcl-2 family were involved in aspisol-mediated apoptosis of MDA-MB-231 breast cancer cells. Up-regulation of bcl-2 by COX-2 may be the mechanism ofthe reduction of apoptotic susceptibility in MDA-MB-231 cells.

In conclusion, it could be assumed that the non-selective COX-2 inhibitor, aspisol, can suppress the viability of MDA-MB-231 cells by induction of apoptosis. This effect of aspisol correlated with down-regulation of COX-2 and bcl-2 expression and up-regulation of caspase-3 expression. Therfore, aspisol should be regarded as the potential chemotherapeutic and cancer preventive agent in human breast cancer prevention/treatment.

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АСПИЗОЛ ИНГИБИРУЕТ РОСТ И ВЫЗЫВАЕТ АПОПТОЗ КЛЕТОК РАКА МОЛОЧНОЙ ЖЕЛЕЗЫ

Нестероидные противовоспалительные препараты ингибируют пролиферацию клеток и вызывают апоптоз во многих опухолевых клеточных линиях, что считается важным механизмом их противоопухолевой активности и профилактики развития рака. Тем не менее молекулярные механизмы апоптотического действия этих препаратов изучены недостаточно. ***Цель:*** **изучить действие неспецифического ингибитора циклогексиназы-2 (COX-2) — аспизола — на злокачественные клетки рака молочной железы *in vitro* и *in vivo*.** ***Методы:*** **выживаемоть клеток MDA-MB-231 определяли с помощью MTT-теста. Апоптотический индекс измеряли с помощью проточной цитометрии и иммуногистохимическим окрашиванием с антителами против COX-2 и каспазы-3. Экспрессию bcl-2 и bax изучали с помощью Вестерн-блот-анализа. Содержание простагландина E_2 (PGE_2) в клетках MDA-MB-231 оценивали методом ELISA.** ***In vivo*** **апоптоз опухолевых клеток определяли путем выявления разрывов ДНК с помощью концевой дезоксинуклеот-идилтранферазы (метод TUNEL).** ***Результаты:*** **показано, что в зависимости от времени инкубации и дозы аспизол угнетал рост клеток MDA-MB-231 *in vitro* и вызывал их апоптоз на фоне повышения экспрессии каспазы-3 и bax, а также снижения экспрессии COX-2 и bcl-2. В условиях *in vivo* аспизол также ингибировал пролиферацию злокачественных клеток рака молочной железы и вызывал их апоптоз.** ***Выводы:*** **данные, полученные *in vitro* и *in vivo*, свидетельствуют о противоопухолевом эффекте аспизола на клетки рака молочной железы, что скорее всего опосредовано его проапоптотическим действием и может быть связано со снижением экспрессии COX-2 и bcl-2, а также повышением экспрессии каспазы-3 и bax.**

Ключевые слова: **аспизол, NSAIDs, апоптоз, COX-2, рак молочной железы.**