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Breast Cancer

RESEARCH ARTICLE

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Blocking of The Prostaglandin E2 Receptor as a Therapeutic Strategy for Treatment of Breast Cancer: Promising Findings in a Mouse Model

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Abstract

Objective: The study aimed to investigate the anticancer effect of E-prostanoid receptor 1 (EP1) antagonist, SC19220, alone or in combination with the COX-2 inhibitor Celecoxob(CXB)® in mice bearing solid Ehrlich carcinoma (SEC). **Methods:** The tumors were induced in 40 female mice, which were divided randomly into four equal groups (n= 10 in each group): Tumor control, CXB, EP1 antagonist, and co-treatment. CXB (10mg/kg) and EP1 antagonist (2mg/kg) were given intraperitoneally every three days, six times in total, then tissue was extracted and prepared for histopathology and measurement of weight, PGE2, and gene expression of EP1 and β 1 integrin. **Results:** Both inhibitors, alone or in combination, showed a significant ($p<0.001$) antitumorigenic effect by decreasing, significantly ($p<0.001$), each of the tumor weights, tumor volumes, PGE2 levels, EP1 and β1-integrin gene expression along with increasing, significantly ($p<0.001$), the P53 tumor suppressor protein. The survival rate was improved from 80% in the control group to reach 100% in the treated groups. The co-treatment by CXB and EP1 antagonist showed a marked decrease in tumor weights and volumes as compared with the single treatment. In parallel, the histopathological findings showed enhanced apoptosis and diminished necrosis in the co-treated group. **Conclusion:** EP1 antagonist proved an antitumorigenic effect alone or combined with CXB and could play a new therapeutic strategy against breast cancer.

Keywords: Breast cancer- CXB- Ehrlich Carcinoma- PGE2- β1-integrin

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Introduction

Breast cancer is one of the most common malignancies worldwide that affects both men and women. In 2020; about two million of all new cancer cases were breast cancers. According to GLOBOCAN 2020; 24.5% of all women worldwide are breast cancer cases. Breast cancer is also the second cause of cancer deaths among women (15.5% of all female cases) (Sung et al., 2021).

Breast cancer development is a multi-step process involving multiple cell types, and its prevention remains challenging (Sung et al., 2021). Cyclooxygenase-1 is a constitutive enzyme that produces prostaglandins to regulate normal physiological processes. Whereas cyclooxygenase 2 (COX2) is induced by cytokines and growth factors to mediate inflammation. The upregulation of COX2 expression may participate in tumorigenesis, cell proliferation, and angiogenesis in different cancer types (Liu et al., 2014). In a model of Ehrlich carcinoma, COX2 has been highly expressed and incorporated in mediating tumor growth and progression (Azab et al., 2020).

Celecoxib (CXB) is a COX2 selective inhibitor that has anti-inflammatory and antipyretic effects. Besides, it has an anticancer effect on breast, lung, and hepatic cancers. The US Food and Drug Administration approved CXB as adjuvant therapy for cancer treatment. Celecoxib exerts its antitumor action by inhibiting proliferation, suppressing the AK strain transforming (AKT) pathway, inducing apoptosis, suppressing angiogenesis and regulating the tumor microenvironment (Li et al., 2018).

Prostaglandin E2 is one of the most important mediators of tumorigenesis that COX2 produces, which activates different pathways like AKT/extracellular signal -regulated kinase (AKT/ERK) in breast cancer tissues and upregulates lymphangiogenesis. PGE2 exerts its effects through four receptors; EP1, EP2, EP3 and EP4 (Nandi et al., 2017; Wang et al., 2018). EP1 activates protein kinase C (PKC), increases focal adhesion kinase (FAK) phosphorylation, and promotes invasion in hepatocellular carcinoma cells (Bai et al., 2013). Additionally, PGE2, by coupling with EP1, plays a role in human cholangiocarcinoma growth by activating the endothelial growth factor receptor, survivin and ERK (Bai

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et al., 2010). SC19220 is a well-known EP1 antagonist that was reported to decrease tumor progression in cancer cell lines, including hepatocellular carcinoma and non-small cell lung carcinoma (Pan et al., 2016).

Based on our findings in our previous in vitro study on MCF7 breast cancer cell line (attached as supplementary file), this in vivo study was conducted to investigate the effect of EP1 antagonist on breast cancer progression in mice bearing (SEC) alone or in combination with COX2 inhibitor (CXB), and to elucidate the possible involved mediators.

Materials and Methods

Drugs

Celecoxib was purchased from Merck® (Taufkirchen, Germany). SC19220 was purchased from Tocris® (Bristol, UK). SC19220 is a dibenzoxazepine hydrazide derivative with the molecular formula: C16H14ClN3O3; Purity: 100% using HPLC. Diethyl ether and dimethyl sulphoxide (DMSO) were purchased from S D Fine-Chem Limited Company® (Mumbai, India). CXB and SC19220 were dissolved in 5% DMSO.

Animals

The study was performed by the guidelines for the care and use of laboratory animals approved by the research ethics committee of the Faculty of Pharmacy, Tanta University. Forty female Swiss albino mice aged between 6-8 weeks and weighing 18-22 g, were purchased from the national research center (NRC) in Cairo, Egypt. Mice were kept for one week in wire cages (22°C) under daylight for acclimatization and were provided with water and a standard pellets eds diet ad libitum. The study was conducted at the animal facility of the Faculty of Pharmacy, Tanta University.

Induction of SEC model

Ehrlich ascites carcinoma (EAC) derived from mouse bearing breast adenocarcinoma is obtained from the Pharmacology and Experimental Oncology Unit of the National Cancer Institute (Cairo University, Giza, Egypt). We implanted 1 × 10^6 EAC cells in a mouse by intraperitoneal injection. An ascitic fluid containing Ehrlich cells was developed within 10 days. Cells were withdrawn by a syringe, diluted with 0.9% sterile saline (1:9 v/v) and counted using a Neubauer Hemocytometer (Sigma Aldrich, St. Louis, MO, USA) (El-Ashmawy et al., 2017). Using the trypan blue dye exclusion method, the cell viability was determined to be more than 99% (Shahabuddin et al., 2011), The SEC model was induced by injecting the viable EAC cells (1x10^6 cells) subcutaneously into the mice's right flank. A palpable solid tumor mass (about 100 mm3) was developed within 12 days (Frajacomo et al., 2016).

Experimental design

We randomly assigned the SEC-bearing mice to four equal groups (n= 10) as follows: Tumor control group (mice received the vehicle, DMSO), CXB group (mice were given 10 mg/kg CXB) (Raut et al., 2004). EP1 antagonist group (mice were given 2mg/kg EP1 antagonist) (Lee et al., 2013) and the co-treated group (mice were given both EP1 antagonist and CXB with the specified doses). Both CXB and EP1 antagonist were given every three days, intraperitoneally, six times in total, starting on the 12th day of SEC induction till the 28th day. On the 28th day, the experiment was ended and all mice were euthanized under anesthesia and tumors were excised at necropsy. Tumor tissues were excised, and carefully divided into portions, one portion was kept in 10% formalin for histopathological examination and the other portions were kept in -80°C for other biochemical measurements.

Measurement of tumor volumes, weights and survival rate

Tumor volume was calculated using the following formula: tumor volume (mm^3) = 0.52 AB2, where A is the length of the minor axis and B is the major axis length (Osman et al., 1993). The dimensions of the tumors were measured with a Vernier digital caliper (Anyi Instrument Co., China) starting on the 12th day, and then day after day till the end of the experiment.

The survival rate was calculated as follows: survival rate = (the number of live animals in a group on the 28th day/number of animals in the same group at the start of the experiment) × 100 (Cosetti et al., 2008). On the 28th day, mice were anesthetized by diethyl ether, and sacrificed by cervical dislocation. Tumor tissue was carefully excised, weighed using a sensitive electrical balance (ADAM®, UK) and kept frozen at −80°C till analysis.

Determination of prostaglandin E2 and P53 levels

Enzyme-linked immunosorbent assay (ELISA) kits obtained from Bio neovan Co. Ltd. Beijing, China) were used to determine PGE2 and P53 levels in the tumor tissue. 100 mg tumor tissue was homogenized in one mL PBS (1 w/10 v). The homogenate was centrifuged at 2,500 rpm for 5 min and the supernatant was obtained to measure PGE2 and P53 levels according to the manufacturer's protocol. The assay ranges for PGE2 and P53 are 0.8-50 pg/mL and 1-80 pg/mL, respectively.

Quantitative Real Time-PCR (qRT-PCR) for determination of β1-integrin and E prostanoid 1 gene expression

RNA was extracted from tumor tissue using RNA-spin total RNA extraction kit Bioer Technology (Hangzhou, China), according to the manufacturer's procedures. 1–5μg of total RNA was used for preparing complementary DNA (cDNA) by HiSenScript RH (-) cDNA synthesis kit (iNtRON Biotechnology Co., Seongnam, Korea). Primers` sequences of β1-integrin, E prostanoid receptor 1 (EP1) and GAPDH (housekeeping gene) were: F: 5`TTCTTATTGGCCTTGCCTTG3`, R: 5`CAGTTGTCACGGCACTCTTG3`, F: 5`TAACCTGAGCCTAGCGGATG3`, R: 5`CCAGCGTCATGGAGAAGATAG3`, F: 5`ATTCAACGGCACAGTCAAGG3`, R: 5`TCTCCATGGTGGTGAAGACA3`, respectively. The primers were designed using Primer3 plus software and purchased from Invitrogen Co., (USA). The obtained cDNA was used for qRT-PCR with SensiFAST SYBR

No-ROX PCR kit (Bioline Co., New Jersey, USA). RT-PCR program was adjusted with initial activation for 2 min at 94°C, followed by 45 cycles (94°C for 5 sec for denaturation, 60°C for 10 sec for annealing and 72°C for 20 sec for extension). The target gene Ct values were normalized to the Ct value of GADPH and expressed as a relative fold change in gene expression. The relative quantitation (RQ) of the target gene was calculated according to the Livak method: $RQ = 2^{-\Delta\Delta CT}$ (Livak et al., 2001).

Histopathology of tumor tissue

Tissues were fixed in 10% formalin and then embedded in paraffin to form blocks. Then, tissues were cut into 5μm sections using Leica microtome (Leica, Germany), mounted on glass slides and stained with hematoxylin and eosin (H&E). The histopathological characterization was examined by light microscopy.

Statistical analysis

For the statistical analysis, the Statistical Package for Social Science (SPSS) version 25 was used (George and Mallery, 2018). Differences between groups were statistically analyzed using a one-way analysis of variance (ANOVA). LSD Post hoc analysis was used. The results were expressed as means ± standard deviation (SD). P values less than 0.05 were considered statistically significant. The correlation between different parameters was conducted using Pearson's correlation analysis.

Results

Effect of treatments on tumor weights, volumes and survival rates

The treated groups had a 20% higher survival rate (100% survival rate) than the untreated control group. Figure 1 shows the effect of treatment with each of the CXB and EP1 antagonist from day 12 to day 28 on mice bearing SEC. Both CXB and EP1 antagonist groups exhibited a significant decrease in tumor weights by 56.04% and 49.5% ($P<0.05$) respectively, compared with the tumor control group. The co-treatment with EP1 antagonist and CXB significantly decreased the tumor weights by 69.9% ($P<0.001$), when compared with the tumor control group. Moreover, the co-treatment group showed a significant decrease ($P<0.001$) in weight by 31.5% and 40.41% compared with CXB and EP1 antagonist, respectively (Figure 1A). In the tumor control group, the tumor volume was 164.5 ± 18.95 mm^3 on the 12th day and increased gradually to reach 2004.11 ± 129.3 mm^3 on day 28. However, CXB, EP1 antagonist, and the co-treatment significantly decreased the tumor volume ($P < 0.001$) on the 28th day to 1,168.3 ± 117.18 mm^3,1,333.75 ± 73.58 mm3 , 964.94 ± 69.62 mm^3, respectively, compared to the tumor control group (Figure 1B).

Effect of treatments on prostaglandin E2 (PGE2) and P53 levels

Solid Ehrlich carcinoma-bearing mice treated with CXB, EP1 antagonist or the co-treatment showed a significant decrease in tumor PGE2 levels by 72.22%, 65.62% and 81.45%, respectively when compared with the tumor control group. Moreover, the co-treatment had 27.01% and 46.06% lower PGE2 levels than the CXB or EP1 antagonist groups, respectively ($P < 0.001$) (Figure 2A).

Treating SEC-bearing mice with CXB, EP1 antagonist or the co-treatment showed a significant increase in tumor P53 levels by 1.66, 1.3 and 2.48 fold, respectively when compared with the tumor control group. Moreover, the co-treatment showed a 1.49 and 1.94 fold increase in P53 levels when compared with the CXB or EP1 antagonist groups, respectively ($P < 0.001$) (Figure 2B).

Effect of treatments on EP1 and β1-integrin gene expression

As shown in Figure 3, the tumoral EP1 gene expressions in the CXB, EP1 antagonist, and co-treatment groups were significantly lower (61%, 41%, and 76.3%, respectively) than in the tumor control group. EP1 gene expression in the co-treatment group was also significantly ($P < 0.001$) lower than those of the mono-treated groups. Moreover, the tumoral β1-integrin gene expressions in the CBX, EP1 antagonist, and co-treatment groups were significantly lower (67%, 44%, and 74%, respectively) than in the tumor control group. β1-integrin expression levels in the co-treatment group were also significantly lower than in the CXB (21.12%, $P < 0.05$) and EP1 antagonist (53.7%, $P < 0.001$) groups.

Histopathological findings

Tumor sections from the control tumor group showed numbers of mitotic figures and necrotic areas (Figure 4. a). The EP1 antagonist treated group showed necrosis, mitotic figure and appearance of apoptotic bodies (Figure 4. b). The CXB treated group showed some apoptotic figures and small necrotic areas (Figure 4 c). The co-treated group showed mitotic figures, decreased area of necrosis and

Table 1. Correlation between Measured Parameters in the Treated Groups

	Tumor weight	Tumor volume	PGE2	β1-integrin	P53	EP1
Tumor weight	1	0.80*	0.88*	0.73*	-0.4*	0.72*
Tumor volume	0.8*	1	0.96*	0.95*	-0.76*	0.96*
PGE2	0.88*	0.96*	1	0.95*	-0.7*	0.95*
β1-integrin	0.73*	0.95*	0.95*	1	-0.83*	0.98*
P53	-0.4*	-0.76*	-0.7*	-0.83*	1	-0.85*
EP1	0.72*	0.96*	0.95*	0.98*	-0.85*	1

*, Correlation is significant at the 0.01 level (2 tailed).

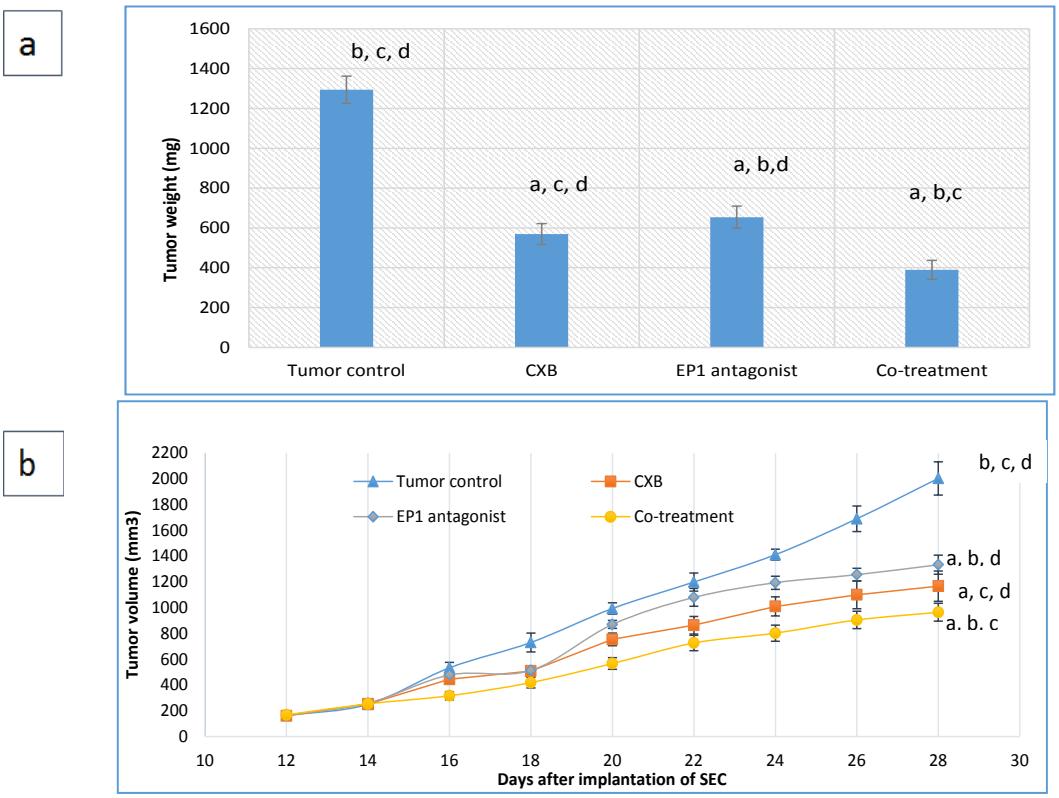

Figure 1. Effect of Celecoxib (CXB) and EP1 Antagonist on Tumor Weight (A), tumor volume (B) in mice bearing SEC. Data are presented as mean ±SD, P<0.05. n= 8. a: Significant versus tumor control, b: Significant versus CXB group, c: Significant versus EP1 antagonist group, d: Significant versus co-treatment group, EP1 antagonist: EP1 antagonist (2mg/kg), CXB: celecoxib (10mg/kg). SD: Standard deviation. Both CXB and SC19220 were given every three days, intraperitoneally, for 6 cycles starting on the 12th day of SEC induction.

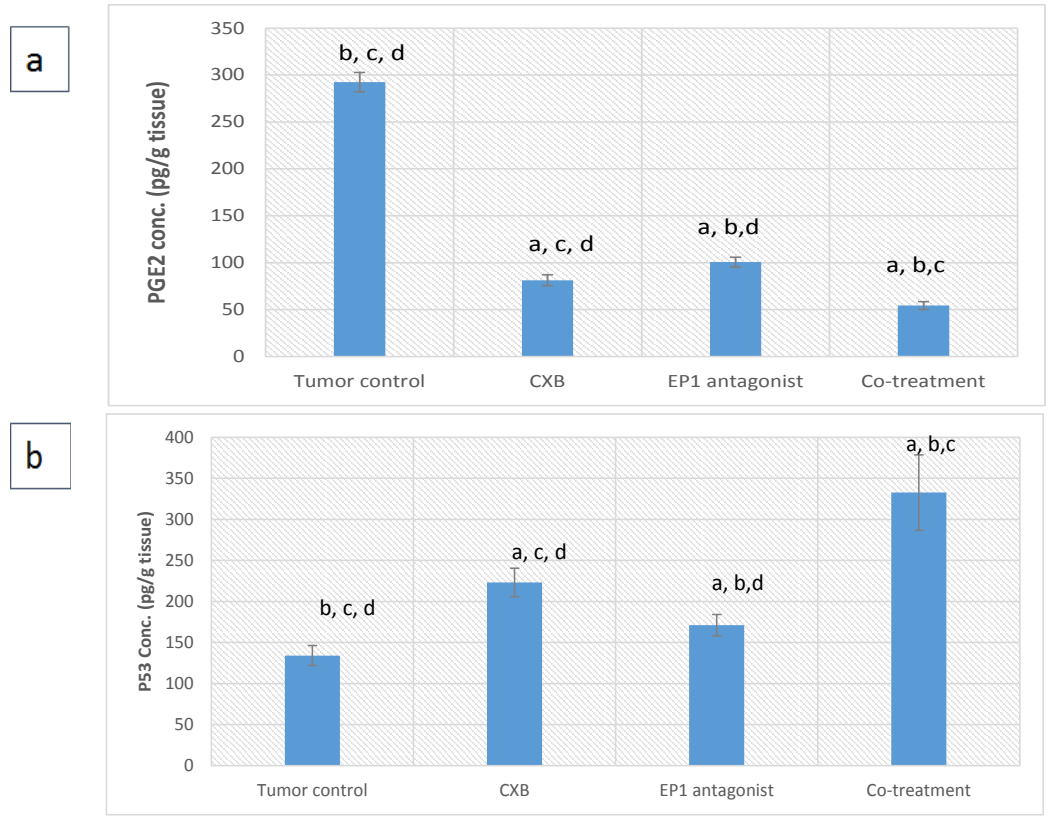

Figure 2. Effect of Treatment with CXB &EP1 Antagonist on PGE2 (A) and P53 (B) Levels in SEC bearing Mice. Data are expressed as Mean±SD, P<0.05. n=8 a: Significant versus tumor control, b: Significant versus CXB group, c: Significant versus EP1 antagonist group, d: Significant versus Co-treatment group. PGE2: Prostaglandin E2. EP1 antagonist (2mg/kg). CXB: CXB (10mg/kg). SD: Standard deviation. Both CXB and EP1 antagonist were given every three days, intraperitoneally, for 6 cycles starting on the 12th day of SEC induction. One way ANOVA was used for experimental analysis

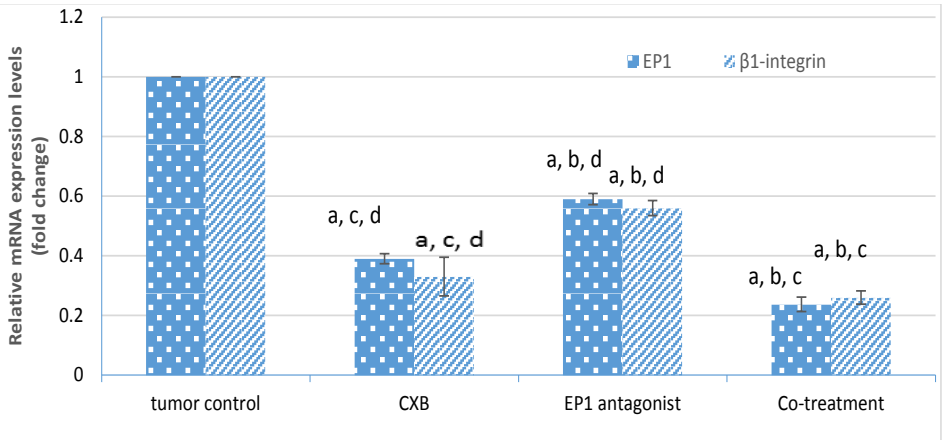

Figure 3. Effect of Treatments on Gene Expression of EP1 and β1-integrin. Data are expressed as mean ±SD, P<0.05 a: Significant versus tumor control, b: Significant versus celecoxib group, c: Significant versus EP1 antagonist group, d: Significant versus Co-treatment group, EP1 antagonist (2mg/kg). CXB (10mg/kg). SD: Standard deviation. Both CXB and EP1 antagonist were given every three days, intraperitoneally, for 6 cycles starting on the 12th day of SEC induction.

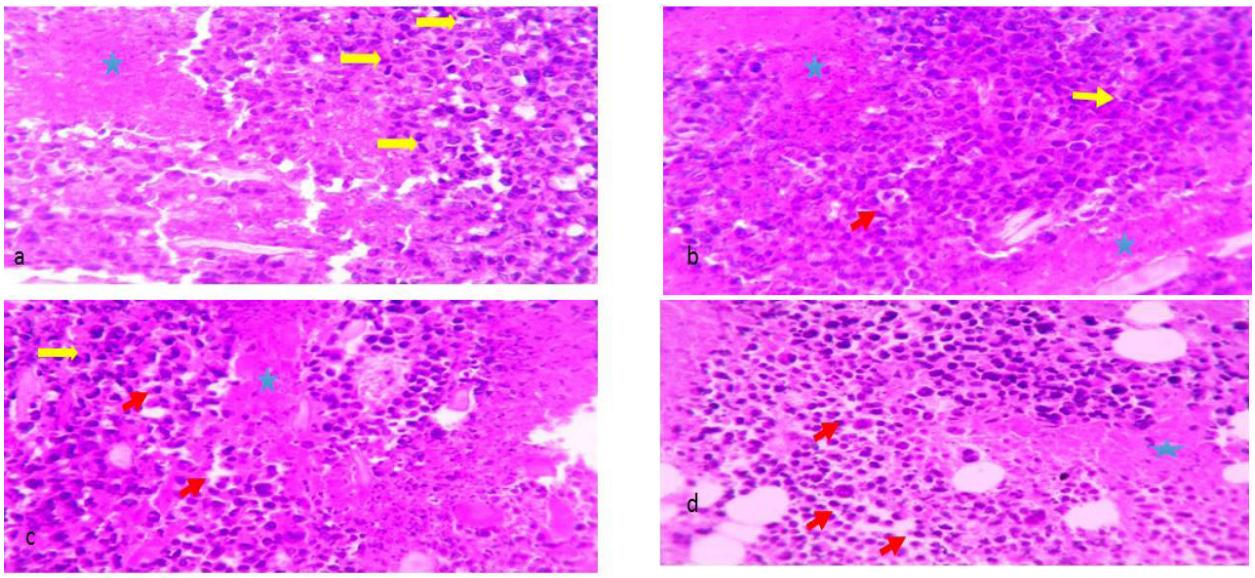

Figure 4. Photomicrographs (H&E) of Tumor Sections in SEC-bearing Mice (magnification= 400X). a) Control tumor group showing many mitotic figures, large, round, and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei, and binucleation and large area of necrosis. b) EP1 antagonist group showing decreased necrotic area (star) and mitotic figures (yellow arrow) with appearance of few apoptotic bodies (red arrow). c) CXB treated group showing decreased areas of necrosis and mitotic figures with presence apoptotic bodies. d) Co-treatment group showing number of apoptotic bodies with small necrotic area and mitotic figures

numbers of apoptotic cells (Figure 4 d).

Correlation study results

Table 1; illustrates correlation between measured parameters in treated groups. A significant positive correlation was found between PGE2 and β1-integrin (r=0.95), EP1 and β1-integrin (r=0.98), β1-integrin and tumor weight (r=0.73), β1-integrin and tumor volume (r=0.95). Moreover, each of tumor weight and volume were positively correlated with PGE2 (r=0.88), (r=0.96), respectively. On the other hand, P53 showed a significant negative correlation with tumor volume, tumor weight, PGE2 and β1-integrin (r=-0.76), (r=-0.4), (r=-0.7), (r=-0.83), respectively.

Discussion

Since chemotherapy has numerous side effects, recently, attention is paid to new treatment strategies focusing on possible pathways involved in cancer development. In the current study, we assessed the effect of the PGE2 pathway on cancer development in mice bearing SEC, as one of the breast cancer models that is characterized by a high and rapid growth rate (Barakat et al., 2015). Herein, the implantation of EAC cells in mice induced solid tumors that increased in volume within the subsequent two weeks. The untreated SEC group (tumor control) showed a gradual increase in both tumors volumes and weights until the end of the experiment. Also,

the histopathological findings of the tumors showed the presence of mitotic figures besides large focal areas of necrosis.

In the current study, treating SEC-bearing mice with CXB has slightly increased the survival rate, increased the tumor P53 protein levels and decreased the tumor weights, tumor volumes, tumor PGE2 levels, as well as EP1 and β1-integrin gene expression in the tumors as compared with the tumor control. The histopathological examination of CXB-treated tumor sections also supported the biochemical findings as it has shown a decreased necrotic areas and appearance of apoptotic bodies. A strong positive correlation between PGE2 and tumor weight and volume in CXB treated group was observed in this study. These results were in line with previous reports, which indicated that CXB has an anti-cancer effect and effectively decreased the growth rate and tumor volume in breast and ovarian cancer (Dai et al., 2012; Suri et al., 2016). Moreover, our findings were in accordance with Huang and his co-workers who stated the CXB's ability to target the breast cancer stem cells by inhibiting serum PGE2 which as a result down-regulated other pathways' activity (Huang et al., 2017).

Celecoxib is an inhibitor of COX-2 that decreases the PGE2-mediated ERK, wingless related-integration site and AKT expression in MCF-7 and MDA-MB-231 cell lines. Moreover, CXB can potentiate phosphatase and tensin homolog and suppress Bcl-2. Therefore, abolishing PGE2 production by CXB can suppress the proliferation, growth and metastasis of many cancer cells (Nandi et al., 2017; Nasry et al., 2018).

Previous reports revealed the impact of β1-integrin overexpression in mediating tumor progression in breast and lung cancer cells (Ritzenthaler et al., 2008; Subbaram et al., 2014, El-Ashmawy et al., 2021). Herein, in the CXB treated group, β1- integrin showed a strong positive correlation with tumor weights, volumes and PGE2. Results from others supported our findings as they revealed down-regulation of β1-integrin expression as a result of interfering with COX-2 expression, by siRNA, in A549, H1299, and LLC cell lines and decreased cell proliferation (Pan et al., 2016).

The EP1 antagonist group revealed a marked decrease in both tumors weights and volumes, an effect that was in alignment with the histopathological observation of the tumor sections, which showed appearance of some apoptotic bodies. Also, the EP1 antagonist might exhibited its antitumorigenic effect, partially, by significantly decreasing PGE2 levels, that contributed to inhibiting the tumor volumes and weights. This effect was further confirmed by a positive correlation between each of PGE2, tumor weights and volumes in this group.

In the current study, EP1 antagonist, also, down-regulated β1-integrin expression. This was in line with the findings of other researchers, who stated that EP1 antagonist mitigates β1-integrin expression by affecting E2F transcription factor 1 (Pan et al., 2016). As illustrated, interestingly, both EP1 and integrin expression were decreased after the treatment with EP1 antagonist, this could be explained, partially, by the ability of β1-integrin itself to increase COX2 and PGE2 production, which in turn affect EP1 expression (Ritzenthaler. Et al., 2008). Therefore, we could tell that the link between EP1 receptor and β1-integrin goes both ways. Supporting this explanation, our correlation results showed a strong positive correlation between each of PGE2, EP1 receptor and β1-integrin expressions.

It is worth mentioning that the co-treatment with CXB and EP1 antagonist showed a superior antitumorigenic effect by decreasing PGE2 level, EP1 and β1-integrin expressions, along with the histopathological findings, than the mono-treated groups. This could be explained by the additional effect of the EP1 antagonist by preventing the remaining PGE2 from activating its receptor.

P53 is a key tumor suppressor that is involved in the malignant transformation in the tumor cells. Once activated, P53 suppresses cancer progression by inducing apoptosis and inhibiting proliferation and survival in breast cancer cells (Muller and Vousden, 2014). In the current study, all treated groups increased P53 protein levels, with greater effect in the co-treated group than in the mono-treated ones. This effect was in accordance with previous studies that reported the ability of CXB to increase P53 levels and decrease tumor progression in chemically-induced colon cancer (Sharaf et al., 2018). In addition, CXB decreased AKT phosphorylation which subsequently enhanced P53 level in acute lymphoblastic leukemia (Naderali et al., 2019; Wen et al., 2020).

Also, all treated groups showed a negative correlation between PGE2 and P53 tumor levels and a negative correlation between P53 and β1-integrin. These results were in line with others that reported a decrease in P53 level could increase β1-integrin and induce cancer proliferation, an effect that could be explained by the ability of P53 to suppress EFGR reuse, which is needed for β1-integrin overexpression in the tumor cells (Pan. Et al., 2018). To the best of our knowledge, this study is the first to study the role of both P53 and β1 integrin and correlate them to the CXB antitumor effect in Ehrlich carcinoma breast cancer.

In conclusion, Celecoxib, a COX2 inhibitor, showed anti-tumorigenic effects by decreasing tumor volume and tumor weight and increasing P53 levels. The anti-tumorigenic effect of CXB was mediated by decreasing PGE2 level, EP1 receptor expression and β1-integrin gene expression in the tumor tissue. Similarly, the EP1 antagonist, decreased tumor volume and tumor weight and increased P53 level, an effect mediated by decreasing PGE2 level and β1-integrin expression. Combined, CXB and EP1 antagonist had a superior effect than alone, thus suggesting a new strategy for breast cancer treatment by targeting PGE2 along with the EP1 receptor.

Limitations

Further biochemical studies on different breast cancer models and clinical investigations should be conducted to put hands on the exact mechanistic mediators involved in breast cancer development.

Author Contribution Statement

Conceptualization: El-Ashmawy N, El-Zamarany E,

Khedr E, Khedr N, Selim H; Supervision and validation: El-Ashmawy N, El-Zamarany E, Khedr E, Khedr N; Investigation, methodology and writing the original draft: Selim H; Resources: El -Ashmawy N; Visualization: Khedr N; Data curation: El-Ashmawy N and Selim H; Reviewing and editing: El -Ashmawy N, El-Zamarany E, Khedr E, Khedr N, Selim H.

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Ethical approval

The study protocol was conducted and confirmed according to the ethical guidelines of the declaration of Helsinki and was approved by the local ethical committee (Tanta University, Faculty of Pharmacy, Egypt) (FPTU-REC, 115/ 2017/620).

Data availability statement

The datasets used during the current study are available from the corresponding author on reasonable request.

Supplementary document

https://www.sciencedirect.com/science/article/pii/S2214750021001396?via%3Dihub

Conflict of interests

The authors declare that they have no conflicts of interest.

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