Estrogen-dependent regulation of Eg5 in breast cancer cells

Maricarmen D. Planas-Silva and Irina S. Filatova

HsEg5 (Eg5) is a kinesin required for proper execution of mitosis. Several compounds that specifically block Eg5 are in clinical development and have the potential to be used in the treatment of breast cancer. In this study, we investigated the interaction between Eg5 and estrogen receptor signaling. We observed decreased Eg5 expression after treatment of estrogen receptor-positive human breast cancer MCF-7 cells with the estrogen receptor downregulator fulvestrant. Downregulation of Eg5 expression in response to fulvestrant was also observed in another estrogen receptor-positive cell line ZR-75, but not in the estrogen receptor-negative breast cancer cell line MDA-231. Moreover, in MCF-7 cells previously arrested in the G_0/G_1 phase of the cell cycle by fulvestrant, addition of estrogen increased Eg5 expression. This upregulation correlated with progression through S-phase. Nevertheless, the effect of fulvestrant in Eg5 expression could not be explained solely by cell cycle arrest, because treatments that blocked cell cycle progression did not consistently decrease Eg5 expression. Pharmacological inhibition of Eg5 function, with either *S*-trityl-L-cysteine or monastrol, prevented growth of estrogen-treated MCF-7 cells with an IC_{50} of 0.46 and 29.71 μmol/l, respectively. Simultaneous inhibition of estrogen receptor function with fulvestrant increased the IC_{50} for *S*-trityl-L-cysteine to 2.30 μmol/l and for monastrol to 112.69 μmol/l. Our results suggest that pharmacological inhibition of Eg5 may be an effective treatment for estrogen receptor-positive breast cancer, even without concomitant hormonal therapy.
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Introduction

Novel therapies to target mitosis are being developed as antineoplastic drugs. Mitotic progression can be blocked not only by disrupting microtubule dynamics, but also by inhibiting the function of other molecules essential for mitosis. One of these molecules is HsEg5 (Eg5), a kinesin-related protein essential for formation of the bipolar mitotic spindle, also known as kinesin spindle protein [1]. Several compounds have been identified that can specifically block Eg5. The first reported small-molecule inhibitor of Eg5 was monastrol, isolated in a phenotype-based screen to discover compounds that block mitosis [2]. Subsequently, other Eg5 inhibitors have been identified and shown to inhibit growth of different tumor cell lines in cell culture models or in-vivo tumor models [3–9].

Eg5 is a potential therapeutic target for the treatment of breast cancer. The treatment strategy for advanced breast cancer depends on whether the tumor cells express estrogen receptor (ER) or not and the hormonal status of the patient [10]. In postmenopausal women, advanced ER-positive breast cancer is currently treated with hormonal therapies such as tamoxifen, which competes with estrogen for binding to the estrogen receptor, or aromatase inhibitors, which block estrogen synthesis [10]. A newer type of hormonal therapy is fulvestrant [11]. Besides acting as an antiestrogen by competing with estrogen for binding to ER, fulvestrant can downregulate ER expression by inducing its degradation [11]. To use Eg5-targeted therapy effectively in ER-positive breast cancer, it is important to determine whether hormonal therapies affect the sensitivity of breast cancer cells to Eg5 blockade. In this study, we evaluated the interaction between Eg5 and ER signaling. To pharmacologically block Eg5 function in ER-positive MCF-7 cells, we used two independent compounds, *S*-trityl-L-cysteine (STLC) [6] and monastrol [2]. We show here that ER function regulates Eg5 expression and the sensitivity of breast cancer cells to Eg5 inhibitors.

Materials and methods

Cell culture

The ER-positive human breast cancer cell lines MCF-7 and ZR-75 were maintained in phenol red-containing Dulbecco's modified Eagle's media (Invitrogen, Carlsbad, California, USA) supplemented with 5% fetal bovine serum (FBS). For hormonal treatments, except when indicated, we used phenol red-free Dulbecco's modified Eagle's media supplemented with 5% charcoal/dextran-stripped fetal bovine serum (CSS) (Hyclone, Logan, Utah, USA).

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Cell growth assays

Cells were plated between 0.7 and 2.5×10^5 cells per well on six-well plates using FBS medium and allowed to recover from trypsinization for 2 or 3 days. At this time (day 0), cells were switched to CSS medium with or without 5 nmol/l 17β-estradiol (E_2), 1 μmol/l tamoxifen or 100 nmol/l fulvestrant (ICI 182,780; Tocris, Ellisville, Missouri, USA) in the presence of solvent control, dimethylsulfoxide or ethanol as appropriate, or different doses of STLC (Tocris) or monastrol (Tocris). On day 3, cells were fixed in 10% trichloroacetic acid and cell growth evaluated using the sulforhodamine B assay [12]. In general, all data points represent the average ± SD obtained from triplicate wells. To calculate IC_{50}, Prism software (Graph Pad Prism v. 4.0, San Diego, California, USA) was used. Values given as IC_{50} represent the average obtained from between two and six independent experiments, depending on specific hormonal treatments.

Cell cycle arrest and rescue

For cell cycle arrest, MCF-7 cells plated in FBS were switched to CSS medium containing 10 nmol/l of fulvestrant for 48 h. At this time ($T = 0$ h), ethanol or E_2 (5 or 50 nmol/l) was added directly to cultures. To evaluate cell cycle progression into S-phase, thymidine incorporation was measured as described previously [13]. For studies to determine the effect of cell cycle arrest on Eg5 expression, cells were treated with the indicated compounds in the presence of 5% FBS.

Flow cytometry

MCF-7 cells were treated with the indicated compounds in the presence of 5% FBS for 48 h. Cells were harvested by trypsinization, fixed in 70% ethanol and stored at 4°C [13]. On the day of the assay, cells were pelleted, the fixative was removed and cells were resuspended in phosphate-buffered saline. Cells were incubated with RNase (BD Biosciences, San Jose, California, USA) (500 μg/ml) followed by propidium iodide added to a final concentration of 50 μg/ml. Cell cycle analyses were carried out with a Becton Dickinson FACScan flow cytometer (BD Biosciences, San Jose, California, USA) and data collected from 25 000 cells was analyzed with ModFitLT (v3.1 Mac) (Verity Software House, Topsham, Maine, USA).

Western immunoblot analyses

Preparation of cell extracts was performed using NP-40-containing buffer, as previously described [13] or, in most cases, by using denaturing conditions [14]. Equal amounts of total protein were resolved on a 4–12% Bis-Tris polyacrylamide gel (Invitrogen) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, Massachusetts, USA) for blotting. Immunoblotting was carried out with antibodies specific for Eg5 (BD Biosciences, Mountain View, California, USA) or β-actin (Sigma-Aldrich, St Louis, Missouri, USA) followed by ECL detection (Amersham Biosciences, Piscataway, New Jersey, USA). For quantification of immunoblots, images were analyzed with Quantity One software (Bio-Rad Laboratories, Hercules, California, USA).

Statistical analyses

Comparisons between two treatments were evaluated using unpaired, two-tailed Student's *t*-test.

Results

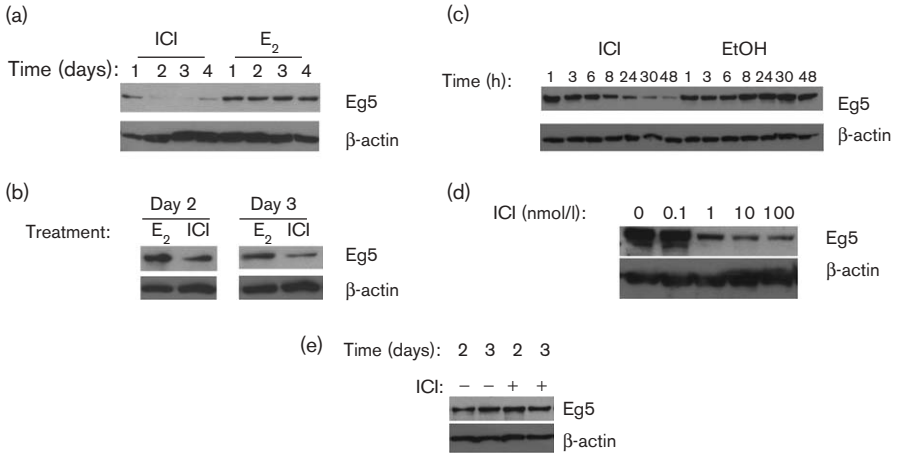
Effect of fulvestrant on Eg5 expression in breast cancer cells

In these studies, we evaluated whether ER function regulates Eg5 expression in MCF-7 cells. It has been shown that all-*trans*-retinoic acid (ATRA), a potent growth inhibitor, can downregulate expression of Eg5 in pancreatic carcinomas and keratinocytes, but not in the ER-positive human breast cancer MCF-7 cell line [15]. Therefore, it was possible that ER function regulates Eg5 expression in MCF-7 cells. To assess the requirement for ER function in MCF-7 cells, we used the ER down-regulator fulvestrant. Asynchronous growing MCF-7 cells were switched to CSS medium containing either 100 nmol/l fulvestrant or 5 nmol/l E_2. Levels of Eg5 expression were determined between 1 and 4 days of treatment. Eg5 expression was maintained throughout the experiment in the presence of estrogen (Fig. 1a). In contrast, Eg5 expression was downregulated at all time points by the presence of fulvestrant. These results suggest that ER function is required for Eg5 expression in MCF-7 cells.

To determine whether Eg5 regulation by ER is observed in other ER-positive breast cancer cells, we tested the effect of fulvestrant in ZR-75 cells. Addition of 100 nmol/l fulvestrant to ZR-75 also decreased Eg5 expression compared with E_2-treated cells (Fig. 1b). These results indicate that the effect of fulvestrant on Eg5 expression is not specific to MCF-7 cells, but is shared by other ER-positive breast cancer cells.

In the previous experiments, to assess the effect of ER downregulation by fulvestrant we had switched cells to CSS medium. CSS, in addition to having decreased levels of estrogen, has decreased levels of other growth factors compared with FBS. Thus, we decided to use FBS to investigate, in detail, the kinetics of Eg5 downregulation during the period between 1 and 48 h of fulvestrant treatment. In addition, to avoid changes in Eg5 expression owing to media replenishment, we fed cells with fresh FBS medium 1 day before addition of fulvestrant or ethanol (control cells). Despite the presence of all the growth factors contained in FBS, fulvestrant again led to a time-dependent decrease in Eg5 expression (Fig. 1c). After 8 h of fulvestrant treatment, levels of Eg5 were 53.5% ($n = 3$) of ethanol-treated cells. By 24 h, Eg5

Fig. 1



Effect of fulvestrant on expression of Eg5 in breast cancer cells. MCF-7 (a) or ZR-75 (b) cells were treated with 100 nmol/l fulvestrant (ICI) or 5 nmol/l 17β-estradiol (E_2) in the presence of 5% CSS. (c) MCF-7 treated with ethanol or 100 nmol/l ICI in the presence of FBS. At the indicated times cells were harvested and analyzed for the expression of Eg5 and β-actin by Western blot. (d) MCF-7 cells in the presence of 5% FBS were treated with the indicated doses of ICI and harvested after 48 h of treatment. (e) MDA-231 cells were treated with 100 nmol/l ICI in the presence of FBS. After 2 or 3 days cells were harvested and analyzed for the expression of Eg5 and β-actin by Western blot. All blots are representative of three or four independent experiments. CSS, charcoal/dextran-stripped fetal bovine serum; ER, estrogen receptor; FBS, fetal bovine serum.

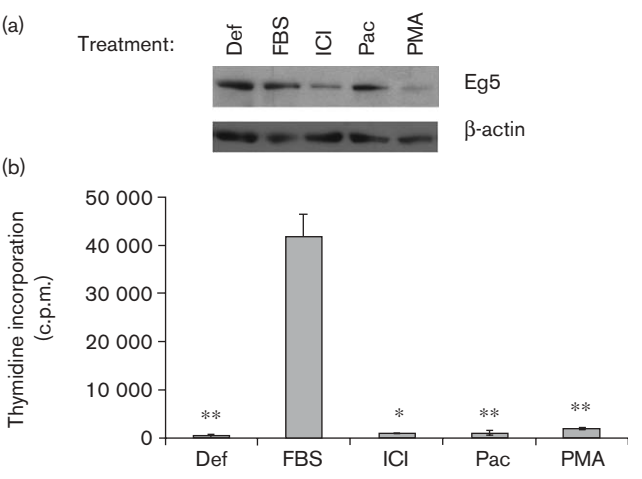
expression in fulvestrant-treated cells was only 26.6% ($n = 3$, $P < 0.05$) of control cells and by 30 h, expression of Eg5 was further reduced to 7.0% ($n = 3$, $P < 0.05$) of control cells. Moreover, changes in Eg5 expression were dependent on the concentration of fulvestrant (Fig. 1d). At 0.1 nmol/l of fulvestrant Eg5 expression was 83.5% of control ($n = 4$, $P < 0.05$) whereas at 10 nmol/l fulvestrant, it was only 14.7% of control values ($n = 4$, $P < 0.05$). Collectively, these results suggest that estrogen signaling is required to maintain Eg5 expression in ER-positive breast cancer cell lines.

To determine whether fulvestrant affects Eg5 expression in ER-negative cell lines, we used the human breast cancer cell line MDA-MB-231 (MDA-231). MDA-231 cells were treated with 100 nmol/l fulvestrant in the presence of 5% FBS for 2 or 3 days. Although treatment with this dose of fulvestrant downregulated expression of Eg5 in MCF-7 and ZR-75 cells, it did not affect Eg5 expression in MDA-231 cells. Therefore, these results suggested that the effect of fulvestrant was dependent on the presence of ER.

Changes in Eg5 expression after cell cycle arrest

Estrogen signaling is required for proliferation of ER-positive breast cancer cells [16]. Conversely, ER downregulation by fulvestrant inhibits proliferation of MCF-7 cells by inducing cell cycle arrest at G_0/G_1 [17]. As the major role of Eg5 occurs during mitotic progression [6,18], it is possible that Eg5 expression is cell cycle regulated and that the decrease in Eg5 expression may result from cell cycle arrest. To evaluate whether cell cycle arrest *per se* causes Eg5 downregulation, we used several pharmacological means to induce cell cycle arrest, such as blocking iron uptake with deferoxamine, stabilizing

Fig. 2



Changes in Eg5 expression after cell cycle arrest. MCF-7 cells growing in 5% FBS were treated with either 25 μmol/l deferoxamine (Def), 100 nmol/l fulvestrant (ICI), 10 nmol/l paclitaxel (Pac), 2 ng/ml PMA for 48 h and compared with control (FBS) for expression of Eg5 and β-actin by Western blot ($n = 6$) (a) or for S-phase progression by thymidine incorporation (b), values represent the average ± SD of triplicate wells from a representative experiment ($n = 4$). *$P = 0.005$, significantly different from control (FBS); **$P < 0.005$. ER, estrogen receptor; FBS, fetal bovine serum; PMA, phorbol myristate acetate.

microtubules with paclitaxel or treatment with a phorbol ester [phorbol myristate acetate (PMA)] known to induce G_1 arrest in MCF-7 cells [19]. Only PMA treatment downregulated Eg5 to the same extent that fulvestrant did (Fig. 2a). A partial decrease of Eg5 expression was observed with paclitaxel, whereas deferoxamine-treated cells exhibited similar levels of Eg5 to control cells maintained in FBS (Fig. 2a). Nevertheless, as judged by thymidine uptake, all these treatments were effective in blocking S-phase progression compared with control cells (Fig. 2b). Thus, downregulation of Eg5 expression by fulvestrant cannot be explained solely by its ability to block S-phase progression and induce cell cycle arrest.

To determine the specific cell cycle phases where cells had arrested we used flow cytometry, as thymidine uptake only indicates whether cells are actively progressing through S-phase or not. The cell cycle profiles of MCF-7 cells treated with the different cell cycle inhibitors for 48 h are shown in Table 1. As expected for fulvestrant-induced cell cycle arrest of MCF-7 cells, most cells (91%) arrested in the G_0/G_1-phase of the cell cycle (*2N* content). Blocking iron uptake in MCF-7 cells, by preventing S-phase progression, arrested most cells (96%) in G_0/G_1 and S-phase, as described [20]. In contrast, PMA arrested MCF-7 cells at G_0/G_1 (63%) and G_2/M (32%) phases, as previously observed [21,22]. As expected for paclitaxel, most MCF-7 cells (74%) were arrested in the G_2/M phase of the cell cycle (*4N* content) after treatment with this drug. No clear correlation, between specific cell cycle arrest and Eg5 expression could be observed. Fulvestrant and PMA were able to reduce Eg5 expression and the percentage of cells present in S-phase as compared with control cells (5% FBS). Fulvestrant, however, only increased the percentage of G_0/G_1 cells relative to control, whereas PMA increased the number of G_2/M cells instead. Although these data cannot clearly indicate that Eg5 expression is cell cycle-regulated, alternative explanations for the effect of PMA in Eg5 expression is the ability of PMA to block estrogen receptor function [23–25] or, perhaps, to induce cell cycle exit (G_0) as fulvestrant does [17].

Table 1 Cell cycle distribution of MCF-7 after treatment with different cell cycle inhibitors

Treatment (*n*)	Cell cycle distribution (%)		
	G_0/G_1	S	G_2/M
Control, 5% FBS (4)	64.10 ± 1.33	24.36 ± 0.75	11.54 ± 1.76
Fulvestrant, 100 nmol/l (4)	91.27 ± 2.38	3.53 ± 0.38	5.21 ± 2.02
Deferoxamine, 25 μmol/l (4)	60.17 ± 8.78	35.34 ± 9.45	4.50 ± 5.73
PMA, 2 ng/ml (3)	62.67 ± 1.35	4.90 ± 0.34	32.43 ± 1.68
Paclitaxel, 10 nmol/l (4)	22.07 ± 3.05	3.53 ± 2.91	74.90 ± 1.90

Cells were treated for 48 h as indicated before measuring cell cycle distribution by flow cytometry as explained in Materials and methods. Values are the mean ± SD of the indicated number of experiments.
FBS, fetal bovine serum; PMA, phorbol myristate acetate.

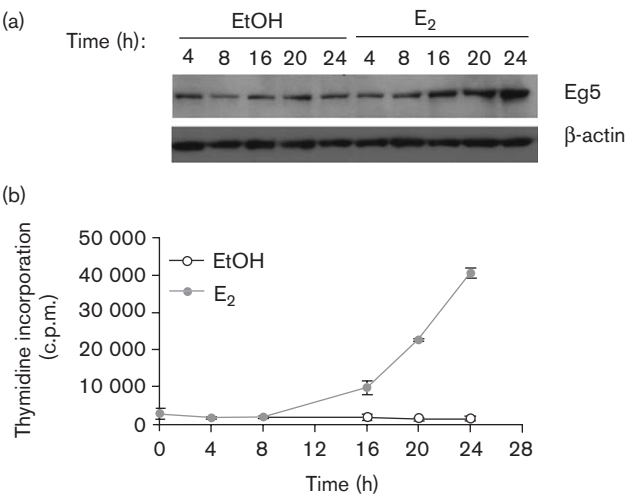
Expression of Eg5 after estrogen-dependent cell cycle re-entry

Fulvestrant-mediated cell cycle arrest can be reversed by the sole addition of estrogen to the arrested cells. We evaluated whether estrogen treatment increases Eg5 expression in cells previously cell cycle arrested by fulvestrant. Addition of E_2 to fulvestrant-treated cells induced Eg5 expression (Fig. 3a). Expression of Eg5 was observable after 16 h of E_2 treatment and peaked at 24 h when it achieved on average ($n = 5$) 11-fold expression over ethanol-treated cells. The highest Eg5 expression coincided with the highest level of thymidine incorporation after estrogen-mediated cell cycle rescue (Fig. 3b). Thus, these results suggest that although estrogen signaling is required for Eg5 expression, additional factors, most likely present during S-phase, may also be needed for Eg5 expression.

Interaction between Eg5 blockade and hormonal therapy

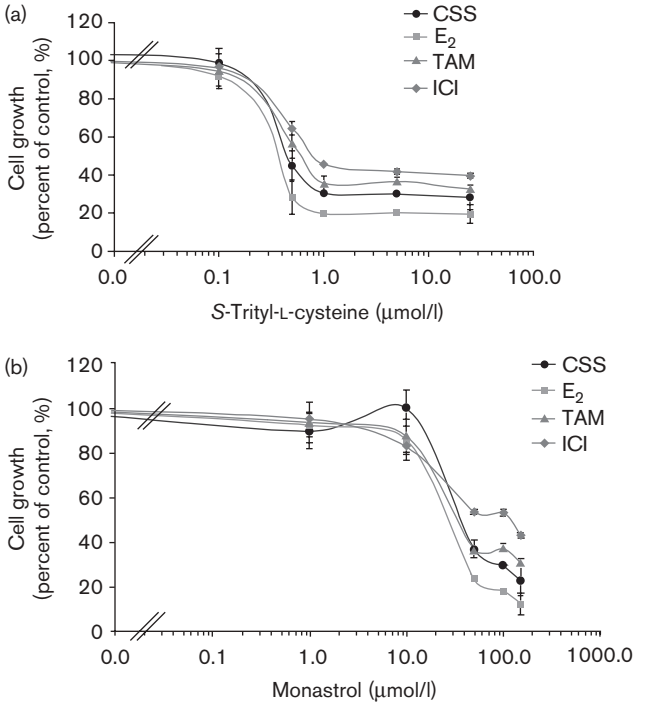
The effect of estrogen in regulating Eg5 expression may influence the ability of Eg5 inhibitors to block proliferation of ER-positive breast cancer cells. To determine the impact of ER signaling in Eg5 blockade, we performed growth assays in the presence or absence of Eg5 inhibitors

Fig. 3



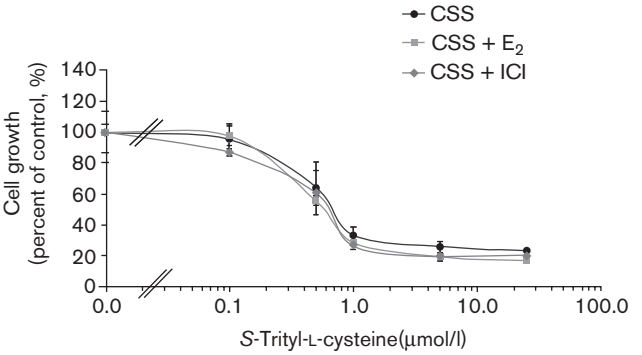
Eg5 expression during estrogen-dependent cell cycle progression. MCF-7 cells were cell cycle arrested by treatment with 10 nmol/l fulvestrant for 48 h. At this time, either ethanol or 50 nmol/l of 17β-estradiol (E_2) were directly added to each well. At the indicated times cells were harvested and analyzed for Eg5 and β-actin expression by Western blot ($n = 4$) (a) or for S-phase progression by thymidine incorporation (b), values represent the average ± SD of two independent experiments.

Fig. 4



Interaction between Eg5 blockade and hormonal treatments in ER-positive MCF-7 cells. Cells were treated with different doses of STLC (a) or monastrol (b) or with either 0.1% DMSO or ethanol, respectively, in the presence of 5% CSS alone (CSS) or with addition of 5 nmol/l of 17β-estradiol (E_2), 1 μmol/l of tamoxifen (TAM), 1 or 10 nmol/l of fulvestrant (ICI). After 72 h of treatment, cells were fixed and processed for SRB as a measure of cell growth. CSS, charcoal/dextran-stripped fetal bovine serum; DMSO, dimethylsulfoxide; ER, estrogen receptor; TAM, tamoxifen; SRB, sulforhodamine B assay; STLC, *S*-trityl-L-cysteine.

Fig. 5



Interaction between Eg5 blockade and hormonal treatments in ER-negative MDA-231 cells. Cells were treated with 0.1% DMSO or different doses of STLC in 5% CSS alone (CSS) or with either 5 nmol/l 17β-estradiol (E_2) or 10 nmol/l fulvestrant (ICI). After 72 h of treatment, cells were fixed and processed for SRB to evaluate cell growth. Results are representative from four or five independent experiments. CSS, charcoal/dextran-stripped fetal bovine serum; DMSO, dimethylsulfoxide; ER, estrogen receptor; TAM, tamoxifen; SRB, sulforhodamine B assay; STLC, *S*-trityl-L-cysteine.

using different hormonal treatments. We compared the effect of Eg5 blockade in MCF-7 cells in the presence of CSS medium and E_2, without added E_2, or in the presence of tamoxifen or fulvestrant. As inhibitors of Eg5 function, we used two commercially available compounds: STLC and monastrol. Growth assays showed that both drugs were effective in blocking growth of MCF-7 cells in the different hormonal milieu (Fig. 4). The maximal inhibitory effect with both compounds, however, was observed with MCF-7 cells proliferating in the presence of E_2. For STLC, the IC_{50} in the presence of E_2 was 0.46 ± 0.18 μmol/l whereas in the presence of fulvestrant it increased to 2.30 ± 0.88 μmol/l. Similarly, fulvestrant treatment increased the IC_{50} for monastrol from 29.71 ± 1.99 μmol/l in the presence of E_2 to 112.69 ± 38.96 μmol/l in the presence of fulvestrant. In contrast, the IC_{50} for STLC were not significantly different in the presence of E_2 or fulvestrant in MDA-231 cells (Fig. 5). Whereas the IC_{50} for MDA-231 grown in the presence of E_2 was 0.86 ± 0.23 μmol/l, in the presence of fulvestrant it was 1.12 ± 0.51 μmol/l. These results suggest that the ability of estrogen to promote cell cycle progression in ER-positive breast cancer cells, by promoting mitosis, may enhance the inhibitory effect of Eg5. Conversely, the decrease in mitotic progression owing to ER blockade may then reduce the effectiveness of Eg5 blockade only in cells that possess ER.

Discussion

The specific role of Eg5 in mitosis has made this kinesin an attractive therapeutic target to block tumor growth by preventing cell cycle progression through mitosis. In this study, we evaluated the interaction between ER function and Eg5 blockade in ER-positive and ER-negative breast cancer cells. We show here that ER function is required for Eg5 expression in ER-positive breast cancer cell lines. Moreover, treatment with the ER downregulator fulvestrant reduces the sensitivity of ER-positive breast cancer cells but not ER-negative cells to Eg5 inhibitors.

As novel therapies targeting Eg5 become available [3–9], it is important to elucidate the regulation of Eg5 expression in human breast cancer cells. We investigated whether ER function affects Eg5 expression. Our findings that Eg5 was downregulated by fulvestrant, even in the presence of growth factors present in FBS, suggested that ER function is required to maintain expression of Eg5 in ER-positive breast cancer cells. As fulvestrant induces cell cycle arrest [17], we, however, also evaluated the effect of other cell cycle inhibitors on Eg5 expression. Our results with compounds that affect cell cycle

progression at different phases of the cell cycle indicate that cell cycle arrest *per se* does not lead to down-regulation of Eg5. Additional evidence to support the idea that growth arrest does not always downregulate Eg5 expression comes from a previous study in which ATRA inhibited growth of MCF-7 cells, but was unable to reduce Eg5 expression [15]. In the same study, the authors showed that several compounds that affected growth of pancreatic cells were unable to reduce Eg5 expression, whereas ATRA was. Thus, it is possible that, depending on the cell type, different nuclear receptors regulate Eg5 expression.

To our knowledge, cell cycle-dependent expression of Eg5 protein in mammalian cells has not been previously reported. Nevertheless, the fact that addition of estrogen to fulvestrant-arrested cells did not immediately induce Eg5 expression but was observed only after 16 h of estrogen treatment when cells progressed through S-phase suggests that Eg5 expression may also be regulated in a cell cycle-dependent manner. In this respect, Eg5 differs from other estrogen-upregulated cell cycle targets such as c-Myc or cyclin D_1 induced several hours after estrogen treatment, well before S-phase progression [16]. Therefore, our data suggest that both estrogen and S-phase factors may be required to promote Eg5 expression in ER-positive breast cancer cells.

The ability of fulvestrant to decrease Eg5 expression prompted us to evaluate whether hormonal therapies affect the ability of Eg5 inhibitors to block cell growth. A recent study using a chemically modified antisense oligonucleotide library revealed that the most effective antisense oligonucleotide to induce 4*N* DNA content in two ER-positive breast cancer cells was directed against Eg5 [26]. Thus, ER-positive breast cancer cells are responsive to Eg5 blockade. As ER-positive breast cancer cells are commonly treated with hormonal therapies it, however, is important to analyze the interaction between hormonal therapies and Eg5 blockade. Using two different small molecules that inhibit Eg5 function, we observed that ER-positive MCF-7 cells, but not ER-negative cells, were more sensitive to Eg5 blockade in the absence of simultaneous ER blockade. These results could be explained by the ability of hormonal therapy to reduce proliferation, and, as a result, decrease mitotic progression and preclude cell cycle arrest by Eg5 inhibitors. In contrast, as estrogen promotes Eg5 expression and proliferation of ER-positive cells, cells growing in the presence of estrogen will be readily growth inhibited in mitosis by Eg5 blockade. If so, these data may indicate that, in general, simultaneous treatment of tumors with Eg5 inhibitors and drugs that block cell cycle progression may reduce the efficacy of Eg5 blockade. In conclusion, our results provide evidence that Eg5 is a promising therapeutic target for proliferating ER-positive breast cancer cells.

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