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Pharmacological inhibition of Rac1-PAK1 axis restores tamoxifen sensitivity in human resistant breast cancer cells

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ABSTRACT

Tamoxifen is a standard endocrine therapy for estrogen receptor positive breast cancer patients. Despite its success, development of resistance mechanisms is still a serious clinical problem. Deregulation of survival signaling pathways play a key role in tamoxifen resistance, being upregulation of Rac1-PAK1 signaling pathway one of the most important. Here, we report the development of the breast cancer cell model MCF7::C1199 having Rac1 enhanced activity with the aim of evaluating the role of Rac1 in acquired endocrine resistance. These cells not only showed distinctive features of Rac1-regulated process as increased migration and proliferation rates, but also showed that upregulation of Rac1 activity triggered a hormonal-independent and tamoxifen resistant phenotype. We also demonstrated that PAK1 activity increases in response to Tamoxifen, increasing phosphorylation levels of estrogen receptor at Ser305, a key phosphorylation site involved in tamoxifen resistance. Finally, we evaluated the effect of 1A-116, a specific Rac1 inhibitor developed by our group, in tamoxifen-resistant cells. 1A-116 effectively restored tamoxifen anti-proliferative effects, switched off PAK1 activity and decreased estrogen receptor phospho-Ser305 levels. Since combination schemes of novel targeted agents with endocrine therapy could be potential new strategies to restore tamoxifen sensibility, these results show that inhibition of Rac1-PAK1 signaling pathway may provides benefits to revert resistance mechanisms in endocrine therapies.

1. Introduction

Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women ranking as the fifth cause of death from cancer overall [1]. It is a highly heterogeneous disease with different biological and histopathological characteristics. Biologically, there are three major subtypes of breast cancer: hormone receptor-positive tumors, which are those expressing estrogen receptors (ER+) and/or progesterone receptors (PR+); ERBB2-amplified tumors, which are those overexpressing the proto-oncogene HER2/neu; and triple-negative tumors (TNBC), which are those that do not express any of those three receptors (ER, PR or HER2/neu) [2].

The best known treatments available (standard of care) are surgery and application of adjuvant therapies as chemotherapy, endocrine therapy and targeting the epidermal growth factor receptors (EGFr) family depending on the tumor type. Hormone receptors positive tumors represent 70% of all cases of breast cancer and endocrine therapy is the most often used adjuvant therapy after surgery for these patients [3]. Endocrine therapy works by decreasing the estrogen levels or by inhibiting the effects of estrogen through the inhibition of ER activity. Tamoxifen (Tam) is a commonly used drug that blocks the effects of estrogen in the breast tissue by attaching to ER in breast cells; it has relatively minor side effects and has been used for >30 years to treat hormone receptor positive breast cancer.

Despite the success of such drug, a significant percentage of patients who initially respond to Tam develop acquired resistance, followed by tumor progression. There are many mechanisms involved in Tam acquired resistance: loss or changes in ER expression, pharmacological mechanisms and upregulation of compensatory signaling pathways, among others [4].

Tam is metabolized to their active metabolites endoxifen and 4-hydroxy-tamoxifen by Cytochrome P450 2D6 (CYP2D6). Modifications in CYP2D6 gene can lead to a reduced activity of this enzyme, producing a decreased effect of Tam [5]. Tumors resistant to Tam generally show overexpression and/or high activity of components of the growth factor signaling pathways. Activation of different receptor tyrosine kinases (RTKs), like EGFr and PI3K/AKT/mTor/MAPK/Erk pathways, among others, have been associated with Tam resistance. The aberrant activity of these signaling pathways could overcome the anti-proliferative

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effects of Tam, compensate its effects and stimulate breast cancer cells growth [6].

Rac1 is a member of the Rho-GTPase family and controls a variety of cellular processes, mainly actin cytoskeleton reorganization, therefore affecting endocytosis and trafficking, cell cycle progression, cell adhesion and migration [7]. More recently has been described that also activate the cellular contractility machinery in a key early window during differentiation to neural stem cells lineage commitment [8,9].

Several studies have suggested that Rac1 pathway is a key player in acquired resistance mechanisms to endocrine therapies, reducing Tam effects. The Rac1 activator breast cancer anti-estrogen resistance gene 3 (AND-34/BCAR3) was identified as a protein involved in the development of endocrine resistance through the activation of the Rac1-PAK1 pathway. Additionally, a constitutively active form of Rac1 caused resistance to hormone treatment in sensitive breast cancer cells [10]. Furthermore, Rac1 inhibition showed anti-proliferative effects in Tam resistant breast cancer cells [11].

PAK1 is the main downstream effector of Rac1 and has been involved in Tam resistance development. PAK1 is able to promote ER phosphorylation at the N-terminal residue Ser305 increasing the expression of Cyclin D1, a gene involved in cancer progression [12,13]. Phosphorylation by PAK1 of ER at Ser305 is one of the main mechanisms associated with Tam resistance.

The role of PAK1 in Tam resistance has also been shown in patients. A cohort of 403 samples from breast cancer patients were evaluated and a correlation between PAK1 high level expression/activation and nuclear translocation was found to contribute to Tam resistance [14,15]. Moreover, a combination of nuclear expression of PAK1 and phosphorylation at Ser305 of ER has been correlated with poor response to Tam in a series of 912 tumors from node-negative breast cancer patients [16].

Aguilar et al. showed that the Vav3-Rac1-PAK1 pathway was linked with endocrine resistance in a genome-wide association study, remarking that the Rac1-PAK1 axis is involved in the development of endocrine resistance [17].

Our group has been studying Rac1 as a therapeutic target, developing pharmacological Rac1 inhibitors in breast cancer and glioblastoma [18,19]. We identified ZINC69391 as a small-molecule that inhibits Rac1 activity by interfering with the interaction between Rac1 and different guanine nucleotide exchange factors (GEFs). With the aim of developing more effective compounds, we carried out a rational design of novel analogues of ZINC69391 and showed that the analogue 1A-116 is a more potent and specific molecule that inhibits Rac1 activation and prevents its effectors activation, i.e. PAK1. Furthermore, 1A-116 showed greater anti-proliferative, anti-invasive and pro-apoptotic effects than the parental compound in several cancer cell lines.

Given the relevance of the Rac1-PAK1 pathway in the development of endocrine resistance and the lack of therapeutic options for patients who do not respond to these therapies, we developed a hormone-independent breast cancer model, called MCF7::C1199, in order to test the role of Rac1 in several mechanisms of acquired endocrine resistance. This model was based on the overexpression of a constitutively active (CA) version of the Rac1 GEF Tiam1 and upregulation of Rac1 activity. Rac1 upregulation promoted a hormone-independent and Tam resistant phenotype. Moreover, Rac1 upregulation caused increased of Ser305 ER phosphorylation levels. The treatment of resistant cells with 1A-116 restored the anti-proliferative effects of Tam, preventing PAK1 nuclear translocation and the subsequent decrease of Ser305 ER phosphorylation. This data suggests that Tam resistance might be prevented or reversed by 1A-116, with the possibility of being useful to restore Tam sensitivity.

2. Materials and methods

2.1. Cell culture and establishment of stable cell lines

For stable transfection and establishment of MCF7 breast cancer cell models, two days prior transfection MCF7 cells (ATCC® HTB-22™) were seeded in 24-wells plates. Transfection was carried out using FuGene HD (Roche Applied Science) in a 4:2 ratio HD, following manufacturer's instructions, with a constitutively active (CA) NH2-terminally truncated C1199 Tiam1 construct, which has been characterized previously [20,21]. The pcDNA.3 empty expression vector was also transfected into MCF7 cells to serve as mock control. Cells were incubated with transfection mixture 8 h, after which the medium was renewed. When reached confluence, cells were trypsinized and plated on T25 flasks in culture medium supplemented with 400 μg/ml G418 (selection medium) (Cat. 11,811,031, ThermoFisher Scientific) and maintained in this medium for about 2 months until stable cells were generated. The cell line expressing the truncated version of Tiam1 was denominated MCF7::C1199 and the control cells were denominated MCF7::pcDNA.3. Both cells lines were grown in Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Sigma-Aldrich Co) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 80 μg/ml gentamicin and 400 μg/ml G418 at 37 °C in 5% CO_2 atmosphere. Cell cultures were routinely subcultured twice a week by trypsinization using standard procedures. For hormone response and anti-proliferative assays, cells were grown in DMEM without phenol red supplemented with 10% charcoal stripped serum (CSS) (Cat. 12676029 ThermoFisher Scientific).

2.2. Drugs

1A-116 Rac1 inhibitor was designed and synthetized as reported in Laboratory of Organic Synthesis, Center of Research and Development in Chemistry, National Institute of Industrial Technology (INTI) [18]. 17-β-Estradiol (E2758) and 4-hydroxytamoxifen (Tam) (H7904) were purchased from Sigma-Aldrich Co. Both were prepared as 1000× stock solutions in absolute ethanol and the corresponding dilutions of ethanol were used as control treatments.

2.3. Western blot

MCF7::pCDNA.3 and MCF7::C1199 cells were plated on 100-mm culture dishes in growth medium. The next day, cells were washed twice with phosphate-buffered saline (PBS). Protein extracts were prepared by homogenizing equal number of cells on ice in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate and 1% NP40) containing protease and phosphatase inhibitor cocktail (P8340 and P0044, Sigma-Aldrich Co). Protein concentrations were determined by Bradford assay and normalized. Samples were separated by SDS-PAGE and analyzed by Western blot. Signals of targets proteins were detected with an enhanced chemiluminescence kit (Kalium Technologies) and the images were taken by C-DiGit Chemiluminescence Western blot Scanner (LI-COR). The following primary antibodies were used: rabbit anti-Tiam1 (sc-872, Santa Cruz Biotechnology, Inc.), rabbit anti-ERα (sc-543, Santa Cruz Biotechnology, Inc.) and rabbit anti-phospho-Estrogen Receptor alpha (Ser305) (05-922R, EMD Millipore).

2.4. Rac1 pull down assay

MCF7::pcDNA.3 and MCF7::C1199 monolayers were washed with PBS and lysed in 150-GPLB lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.5% NP40, 10% glycerol, pH 7.4) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich Co). Lysates were clarified and protein concentrations were determined by Bradford assay and normalized. An aliquot was removed for determination of total Rac1 and the rest was incubated with Glutathione Sepharose 4B Beads (GE Healthcare) coupled with bacterially expressed GST-PAK1. Bound complexes were washed with lysis buffer, resuspended with 4× sample buffer containing β-mercaptoethanol, boiled for 5 min and separated by SDS-PAGE. Proteins were transferred and blotted with mouse monoclonal antibody anti-Rac1 (Sigma-Aldrich Co). Target protein was detected by enhanced chemiluminescence kit (Kalium

Technologies) and images were taken by C-DiGit Chemiluminescence Western blot Scanner (LI-COR).

2.5. Cell proliferation assays

5×10^3 MCF7::pcDNA.3 and MCF7::C1199 cells were plated in 96-wells plates and 24 h later were treated for 72 h with different concentrations of 17-β-Estradiol to evaluate hormone response. To evaluate the reversion of Tam resistance by 1A-116, MCF7::C1199 cells were treated with Tam (0.01 μM, 0.1 μM and 1 μM), 1A-116 (4 μM) or combination of both for 72 h. Cell growth was measured by colorimetric crystal violet assay. The analysis of hormone-dependent growth and Tam resistance reversion was determined using PRISM 6, version 6.01 (GraphPad Prism6® Software Inc., La Jolla, CA, USA). Results shown correspond to the average of three independent experiments.

2.6. Sub-cellular fractioning

Crude subcellular fractionation of MCF7::C1199 cells was performed using different buffers of increasing stringency, as described by Holden et al. [22]. Briefly, MCF7::C1199 cells were plated on 6-wells plates in growth medium and next day media was replaced with DMEM without phenol red supplemented with 10% CSS and treated with Tam (1 μM) alone or in combination with 1A-116 (4 μM) for 24 and 48 h. Subcellular fractioning was performed using digitonin buffer for cytosolic fractions (prepared with 150 mM NaCl, 50 mM HEPES pH 7.4 50 mM and 25 μg/ml digitonin (D141, Sigma-Aldrich Co), and RIPA buffer (for nuclear fractions). Samples were separated by SDS-PAGE. Proteins were transferred and blotted with anti-PAK1 (ab40852, Abcam) and anti-phospho-PAK1 (ab40795, Abcam). Anti-β-Tubulin (ab6046, Abcam) was used for cytosolic loading control and anti-Lamin B1 (ab133741, Abcam) for nuclear loading control. Target proteins were detected by enhanced chemiluminescence kit (Kalium Technologies) and images were taken by C-DiGit Chemiluminescence Western Blot Scanner (LI-COR).

2.7. Measurement of estrogen receptor phosphorylation by flow cytometry

The phosphorylation status of estrogen receptor at Ser305 was measured by flow cytometry using a modified version of the method described by Chow et al. [23]. Briefly, MCF7::C1199 cells were treated with Tam (1 μM), 1A-116 (4 μM) or combination of both for 24 h. Cells suspensions were fixed using paraformaldehyde 4% at room temperature. Following fixation, samples were resuspended in 90% cold methanol in PBS for 15 min at −20 °C. Then, samples were washed in PBS. Finally, cells were labeled with the primary anti-phospho-Estrogen Receptor alpha (Ser305) (05-922R, EMD Millipore) 30 min at 4 °C, washed in PBS and labeled using a FITC-conjugated anti-rabbit IgG secondary antibody (AP132F, EMD Millipore). Cells were washed in PBS and acquisition was achieved by a FACScalibur flow cytometer (Becton Dickinson), and analyzed by FlowJo software. The relative geometric mean fluorescence intensity (gMFI) was calculated by dividing gMFI units of each treatment by gMFI units of control treatment (normal levels of phospho-Ser305 ER) staining in each sample, and expressed as percentage of control. Results shown correspond to the average of three independent experiments.

2.8. Statistical analysis

Statistical analysis was evaluated using PRISM 6, Version 6.01 (GraphPad Prism6® Software Inc., La Jolla, CA, USA). Tukey's Multiple Comparison Test was performed after one-way ANOVA. Two-way ANOVA followed mean 95% confidence interval (CI 95%) after normal distribution of data was confirmed using the Shapiro-Wilk normality test. In addition, the homoscedasticity was determined with Bartlett's test. Differences were considered statistical significant at $p < 0.05$.

3. Results

3.1. Development of a human breast cancer cell model with Rac1 enhanced activity

To explore the relationship between Rac1 regulation, hormone-independence and Tam resistance in breast cancer cells, we generated two cell lines as experimental models by transfecting MCF7 hormone-dependent breast cancer cell line with a CA NH2-terminally truncated C1199 Tiam1 construct (or the empty vector (pcDNA.3).

After G418 selection, we established the model MCF7::C1199 which overexpresses the described truncated version of Tiam1 (Fig. 1a) and effectively has enhanced Rac1 activity (Fig. 1b). As demonstrated by Adam et al., this overactivation caused phenotypic changes in MCF7::C1199 cells associated to Rac1-regulated processes, like lamellipodia formation, associated with increased migratory capacity [24]. Also MCF7::C1199 cell proliferation rate was increased, displaying a lower doubling time in comparison with the control ones (Table 1).

3.2. Rac1 upregulation triggers an estrogen-independent phenotype in breast cancer cells

In order to explore the relationship between Rac1 activity and ER expression we first evaluated ER levels in both MCF7::pcDNA.3 and MCF7::C1199 cells by Western blot (Fig. 2a) and flow cytometry (Fig. 2b). Rac 1 did not altered basal expression levels of ER in our models. To evaluate the response to estrogen *stimulus*, MCF7::pcDNA.3 and MCF7::C1199 cells were treated for 72 h with different concentrations of 17-β-Estradiol and cell proliferation was analyzed. As shown in Fig. 2c, such treatment in MCF7::pcDNA.3 cells increased cell proliferation in a concentration-dependent manner. However, MCF7::C1199 cells did not respond to the same treatment, showing that Rac1 upregulation triggers an estrogen-independent phenotype.

3.3. Inhibition of Rac1 restores Tam sensibility on breast cancer resistant cells

As expected, Tam was able to inhibit MCF7::pcDNA.3 cell proliferation capacity in a concentration-dependent manner, whereas MCF7::C1199 did not show any changes in its proliferation capacity under Tam treatment (Fig. 3a). In order to evaluate the implication of Rac1 in the development of Tam resistant phenotype, we first evaluated the effect of 1A-116 treatment in our models. 1A-116 is a specific Rac1 inhibitor developed by our group with anti-proliferative, anti-invasive and pro-apoptotic effects in several cancer cell lines. 1A-116 showed lesser effect on MCF7::pcDNA.3 cells than on MCF7::C1199 cells. MCF7::C1199 cells showed an IC50 value of 9 μM, a 7-fold reduction when compared to control cells (Fig. 3b). Therefore, we tested different concentrations of Tam in combination with 1A-116. MCF7::C1199 cells

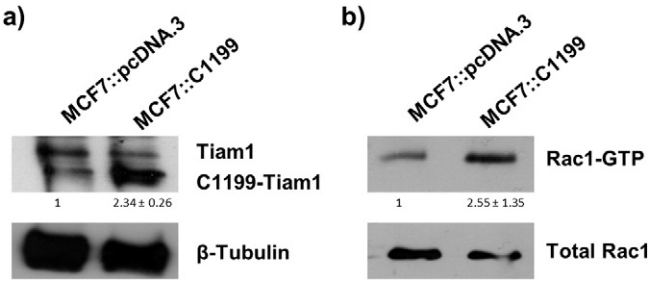

Fig. 1. MCF7::C1199 overexpresses truncated version of Tiam1 (C1199) and has enhanced Rac1 activity. MCF7::pcDNA.3 and MCF7::C1199 cell lysates were separated by SDS-PAGE. a) C1199-Tiam1 levels were detected by Western blot. b) Pull down assays were carried out to detect Rac1 activation levels and then analyzed by Western blot. Densitometric mean ± S.E.M values are shown below (arbitrary units taking MCF7::pcDNA.3 protein levels as 1).

Table 1
Doubling times of MCF7 cells models.

Cell line	Doubling times (h) (mean ± SD)	Growth rate (mean ± SD)
MCF7::pcDNA.3	23.68 ± 2.120	0.0293 ± 0.0027
MCF7::C1199	17.79 ± 0.2851**	0.0388 ± 0.0020**

Unpaired t test, **p < 0.01 vs. MCF7::pcDNA.3.

treated with 1A-116 restored Tam sensibility and its anti-proliferative effects, showing that Rac1 inhibition could reverse Tam resistance (Fig. 3c).

3.4. Inhibition of Rac1-PAK1 signaling pathway reverts Tam resistance mechanisms in resistant breast cancer cells

Several pathways have been proposed to explain Tam-acquired resistance so far and phosphorylation of ER at Ser305 by PAK1 is suggested to be one of the most important mechanism [16,25–27]. We first evaluated PAK1 phosphorylation in response to 1A-116 in MCF7::C1199. 1A-116 treatment decreased phospho-PAK1 levels in a time-dependent manner (Fig. 4a). Then, we evaluated the effect of Tam treatment in phospho-PAK1 levels. As shown in Fig. 4b, Tam treatment increased phospho-PAK1 levels in a time-dependent manner and the presence of 1A-116 reverted the PAK1 phosphorylation induced by Tam (Fig. 4b). Moreover, as shown in Fig. 4c, MCF7::C1199 cells showed phospho-PAK1 presence in nuclear fractions and 1A-116 impaired this nuclear translocation.

Phosphorylation at Ser305 of ER has been proposed as a marker for reduced response to Tam treatment, implicating that inhibition of this activation site could be associated with therapeutic benefits to Tam-resistant patients. Therefore, we first evaluated phospho-Ser305 levels in both MCF7 cell models. We demonstrated by Western blot and flow cytometry that Rac1 upregulation in MCF7::C1199 cells caused increased of Ser305 ER phosphorylation levels (Fig. 5a and b). Consequently, we evaluated the effects of Tam and 1A-116 in Ser305 phosphorylation. As shown in Fig. 5c, phospho-Ser305 levels were increased in response to Tam and the presence of 1A-116 effectively reverted Rac1-PAK1-mediated ER phosphorylation at Ser305.

4. Discussion

Two thirds of breast cancer cases express ER. Hormonal therapy, also called anti-estrogen therapy, works by lowering the amount of estrogen in the body or blocking estrogen from attaching to the breast cancer cells. Endocrine therapy is a first-line hormonal treatment of ER-positive breast cancer [28]. Endocrine therapy involves the use of different drugs depending on breast cancer stage, the different phases of menopause, concomitant diseases, etc. The main types of hormonal therapies that may be used include aromatase inhibitors, which affect estrogen synthesis and decrease its levels; estrogen-receptor downregulators

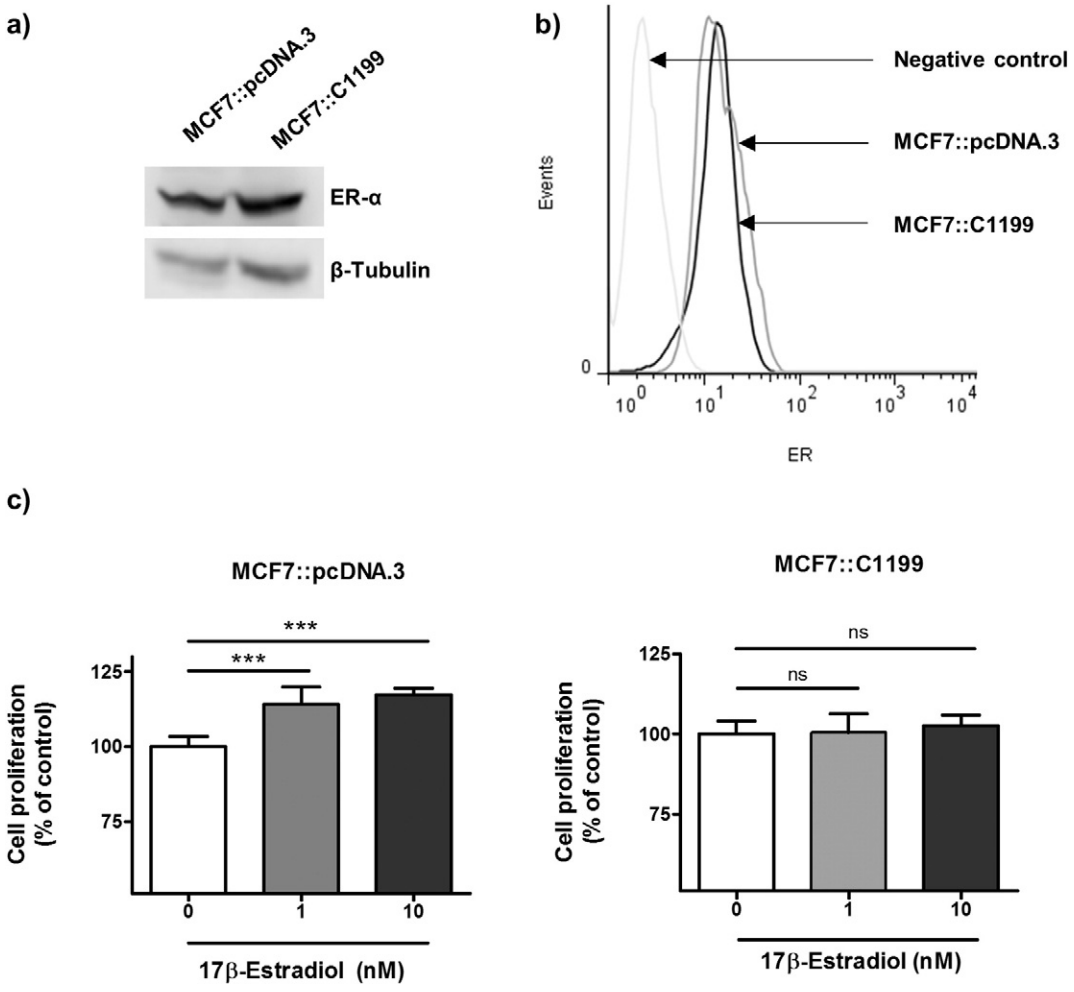

Fig. 2. Rac1 upregulation triggered an estrogen-independent phenotype. ER expression levels in MCF7::pcDNA.3 and MCF7::C1199 cells were detected by a) Western blot and b) flow cytometry. c) MCF7::pcDNA.3 and MCF7::C1199 cells were grown in 96-wells plates and after 24 h were treated with different concentrations of 17-β-Estradiol. Cell viability was measured using colorimetric crystal violet assay. Error bars, S.E.M. ANOVA, Tukey's Multiple Comparison Test; ns, no significant; *p < 0.05; ***p < 0.001 versus control. Results are expressed as percentage of control and are representative of at least three independent experiments.

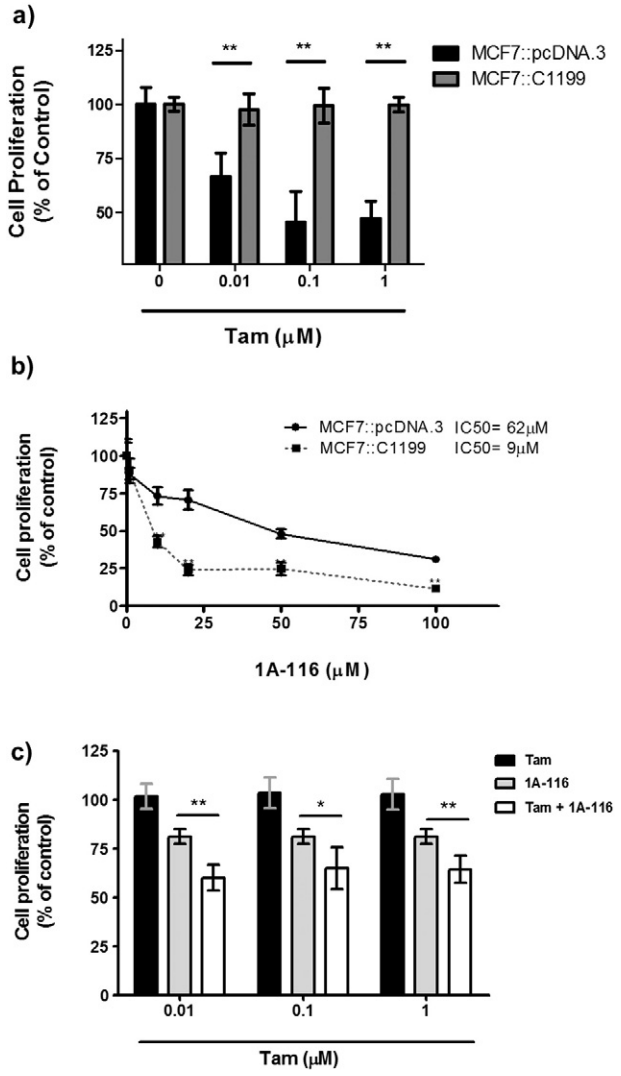

Fig. 3. 1A-116 restores Tam sensibility on MCF7::C1199 resistant cells. a) MCF7::pcDNA.3 and MCF7::C1199 cells were treated for 72 h with different concentrations of a) Tam and b) 1A-116. The concentration producing 50% inhibition (IC50) was determined by non-linear regression function of GraphPad Prism5®. Two way ANOVA Tukey's Multiple Comparison Test, CI 95% comparison, $^{**}p < 0.01$, MCF7::pcDNA.3 vs. MCF7::C1199 at each concentration of 1A-116. C) MCF7::C1199 resistant cells were treated with different concentrations of Tam (black bars), 1A-116 (grey bars, 4 μM) or combination of both drugs (white bars). Concentration of Tam alone or in combination with 1A-116 are listed below each bar group. Error bars, CI 95%, two way ANOVA Tukey's Multiple Comparison Test, CI 95% comparison, $^{*}p < 0.05$, $^{**}p < 0.01$, 1A-116 vs. 1A-116 + Tam at each concentration of Tam. Results are expressed as percentage of control and are representative of at least three independent experiments.

(ERDs) as fulvestrant [29], luteinizing hormone-releasing hormone agents (LHRHs) which shut down the ovaries and stop them from producing estrogen, meaning less estrogen is available to help support the growth of hormone-receptor-positive breast cancer. Examples include goserelin, leuprolide and triptorelin [30] and selective estrogen receptor modulator (SERMs) like Tam, which binds ER blocking its action [4]. Tam is the oldest and most prescribed SERM, since its approval in 1998; Tam has been used to treat millions of women and men diagnosed with estrogen receptor positive breast cancer.

ER signaling pathway comprises a very complex network that regulates gene expression in different ways. The classical or direct genomic pathway involves estrogen-bound ER that promotes proliferation and survival gene expression, like MYC or cyclin D1, that bind to specific DNA regions known as estrogen response elements (EREs) [31]. A non-classical mechanism of genomic action is the ligand-dependent pathway involving the tethering of ER via protein-protein interactions with transcription factors which function as coregulators and thus affect transcription of genes that do not harbor EREs by indirect DNA binding [32]. In the absence of estrogen, a ligand-independent pathway could be activated involving the interaction of ER with other transcription factors to enhance gene expression. Also non-genomic pathways have been described whereas different RTKs can lead to ER activation in the absence of estrogen by protein-protein interactions. Then, ER could activate different cytosolic proteins or transcription factors [4,33].

Although Tam has been successfully used for >30 years to treat ER positive breast cancer tumors, the major clinical problem in the treatment of these patients is the development of resistance mechanisms. A variety of them have been proposed, including decreased or loss of ER expression, increased activity of ER co-activators, post-translational modifications of ER (phosphorylation, methylation and ubiquitination) and increased activity of RTKs including epidermal growth factor receptor (EGFR), HER2 (ERBB2) and insulin-like growth factor receptor (IGFR) leading to deregulation of different signaling pathways.

Because of its complexity the ER signaling pathway presents several possible mechanisms of resistance. Thus, modifications in breast cancer cells that promote progression from hormone-dependent to hormone-independent growth alter their response to endocrine therapy [34].

Rac1 is a small GTP-binding protein of the Rho-GTPase family that mainly regulates the actin cytoskeleton organization, affecting different cellular processes like adhesion, migration, gene transcription and cell cycle progression, among others. Numerous studies reported that aberrant Rac1 activation results in pathological effects not only in carcinogenesis, but also in neuronal disorders and cirrhosis [7,35]. There are at least three characteristics that may contribute to increased activity of this protein: genetic alterations, overexpression and altered activation/inactivation cycles. Although it has been described a common mutation in Rac1 gene in melanoma due to sun exposure [36,37], this mutation has not been found in breast cancer. Moreover, deregulation of Rac1 activation cycle by GEFs can contribute to malignant transformation [38].

Rac1 signaling pathway has been proposed to be involved in the development of acquired resistance to endocrine therapies. ER positive breast cancer cells expressing a constitutively active form of Rac1 induce antiestrogen resistance. In line with this evidence, overexpression of the GDP exchange factor AND-34/BCAR3, and subsequently its Rac1-PAK1 signaling pathway activation, triggered antiestrogen resistance in breast cancer cell lines [10]. Other studies showed that Rac1 inhibition had anti-proliferative effects in ER positive/Tam resistant breast cancer cells [11].

In the present study, we developed the cell line MCF7::C1199 by transfecting the hormone-dependent breast cancer cell line MCF7 with a constitutively active NH2-terminally truncated version of the Rac1 GEF Tiam1 (C1199). The rationale behind this strategy was to develop a breast cancer cell model with Rac1 upregulated activity to explore several Rac1-dependent mechanisms in acquired endocrine resistance. MCF7::C1199 cells overexpresses Tiam1 C1199 protein and have Rac1 upregulated activity. Moreover, MCF7::C1199 cells displayed phenotypic features distinctive of Rac1-regulated processes, like increased lamellipodia formation and migration. In line with studies showing that Rac1 is able to promote G1 phase progression by activating cyclin D1 an increased proliferation rate was observed [39].

The presence of ER is a determinant to choose endocrine therapies as adjuvant treatment. MCF7::C1199 cells did not show changes in the ER expression levels, in comparison with mock control. However, MCF7::C1199 cells are unresponsive to 17-β-Estradiol stimulus, showing that upregulation of Rac1 signaling pathway is able to promote a hormone-independent phenotype. This Rac1 enhanced activity not only promotes a hormone-independent phenotype but also impacts negatively in Tam treatment hence, MCF7::C1199 exhibited Tam resistance.

As mentioned above, in cases where Tam treatment is initially effective, eventually other signaling pathways are activated and Tam

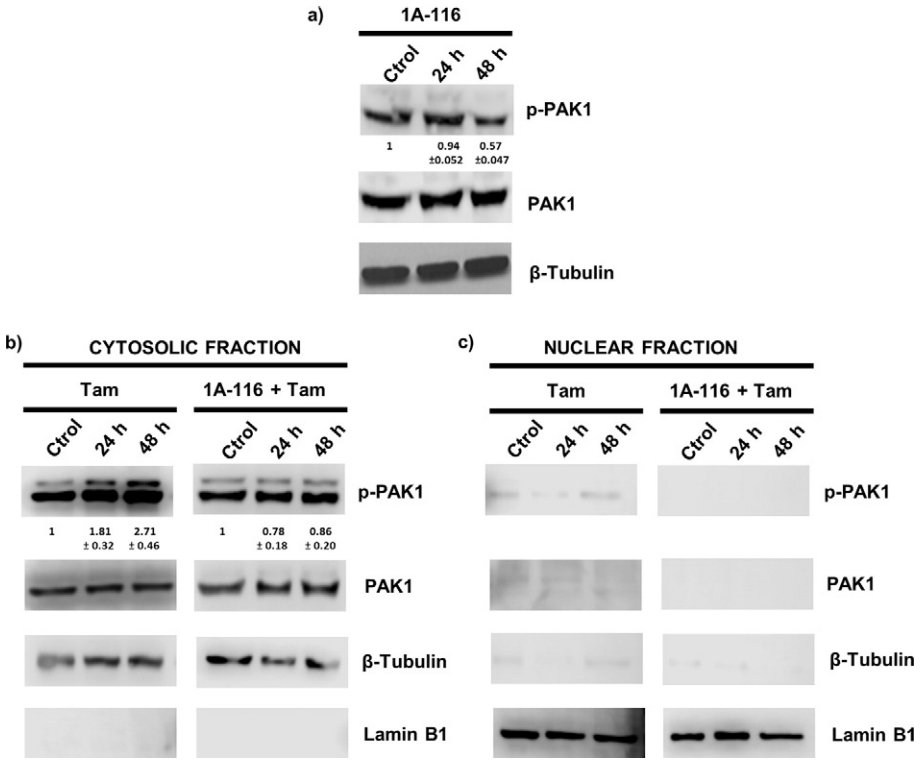

Fig. 4. PAK1 phosphorylation increased in response to Tam and its nuclear translocation is reverted by 1A-116. MCF7::C1199 cells were treated with a) 1A-116 (4 μM), b–c) Tam (1 μM) or combination of 1A-116 (4 μM) + Tam (1 μM) for different times. Subcellular fractioning was performed to generate b) cytosolic and c) nuclear fractions. Phospho-PAK1 and PAK1 levels were detected by Western blot. Densitometric mean ± S.E.M values of phospho-PAK1 relativized to PAK1 total levels are shown below (arbitrary units taking control treatment as 1). Anti-β-Tubulin and anti-Lamin B1 were used as loading control of cytosolic and nuclear fraction, respectively.

becomes ineffective. Once tumors develop Tam resistance, they acquire a more aggressive phenotype that correlate with cancer progression and poor clinical prognosis [40]. For this reason, the combination of novel targeted agents with established endocrine therapy could be an alternative approach for acquired Tam-resistance in breast cancer.

To date, there are many combination strategies based on intracellular signaling pathways inhibitors in breast cancer (PI3K, AKT, mammalian target of rapamycin –mTOR–, ERK). Everolimus received the first approval from the class of drugs known as mTOR inhibitors for the treatment of ER positive, HER2-negative metastatic or locally advanced breast cancer. However, many other signaling pathways inhibitors are being evaluated in several clinical trials with promising results [6].

In previous studies, we reported the antitumoral effect of 1A-116, a novel Rac1 inhibitor developed by our group [18,19]. 1A-116 is a specific Rac1 inhibitor with anti-proliferative and anti-metastatic activities in aggressive breast cancer cells. 1A-116 is well-tolerated and preliminary pharmacological studies showed that the therapeutic dose tested in vivo is approximately 20-times lower than the median lethal dose (LD50). In agreement with observations that Rac1 inhibition could revert Tam resistance [11], the combination treatment of 1A-116 with Tam showed anti-proliferative effects. Our data indicate that treatment with 1A-116 in combination with Tam restores the anti-proliferative activity of Tam, resulting on, a priori, cooperative inhibition of proliferation. These results encourage further in vitro and in vivo testing, also in different combination schemes to evaluate synergistic activity between these two drugs.

The serine/threonine-protein kinase PAK 1 is one of the main downstream effectors of Rac1 and links Rac1 activity with several signal transduction pathways. PAK1 overexpression and/or overactivation increased migration and invasion of breast cancer cells and plays a key role in cell transformation by promoting cell survival and proliferation of many different cancer types, such as breast, prostate, ovarian and colon cancer. PAK1 expression levels correlate with aggressive breast cancer tumors. Moreover, PAK1 is overactivated in about 50% of human breast cancer cases [41].

An increasing body of evidence indicates that PAK1 is involved in the development of Tam resistance in breast cancer. PAK1 activation and its nuclear translocation have been associated with poor prognosis and reduced response to Tam, showing that PAK1 could be a predictor of recurrence and Tam resistance [15,42–44]. In agreement with these observations, we demonstrated that MCF7::C1199 resistant cells increased cytosolic PAK1 phosphorylation levels in response to Tam. Treatment with 1A-116 switches off PAK1 activation and prevented nuclear translocation, without affecting PAK1 expression levels.

Upregulation of Rac1-PAK1 signaling pathway is a very important mechanism associated with Tam resistance. PAK1 is able to phosphorylate the N-terminal residue Ser305 of ER and promote hyperplasia in mammary epithelium [45]. ER phosphorylation at Ser305 by PAK1 could also transactivate ER at residue Ser118, promoting hormone independence and Tam resistance [12,46]. Phosphorylation by PAK1 at Ser305 is the only site that has been correlated with Tam resistance [47]. Furthermore, there is strong clinical evidence that nuclear expression of PAK1 and phosphorylation at Ser305 are associated with poor response to Tam [14,16,25,48]. This association between PAK1 localization and Ser305 phosphorylation is independent of the clinical grade or the hormonal status of the patients.

Phospho-Ser305 confers a unique conformational arrest of ER where Tam can still bind, but failed to promote its inactive form [49]. In line with this observation, MCF7::C1199 resistant cells showed increased levels of phospho-Ser305, in comparison with MCF7::pcDNA.3 control cells, showing that Rac1 upregulation induced increased phospho-Ser305 levels. It is important to highlight that Ser305 phosphorylation was increased in response to Tam treatment. Inhibition of Rac1-PAK1 axis by 1A-116 reverted this resistance mechanism, restoring phospho-Ser305 to normal levels.

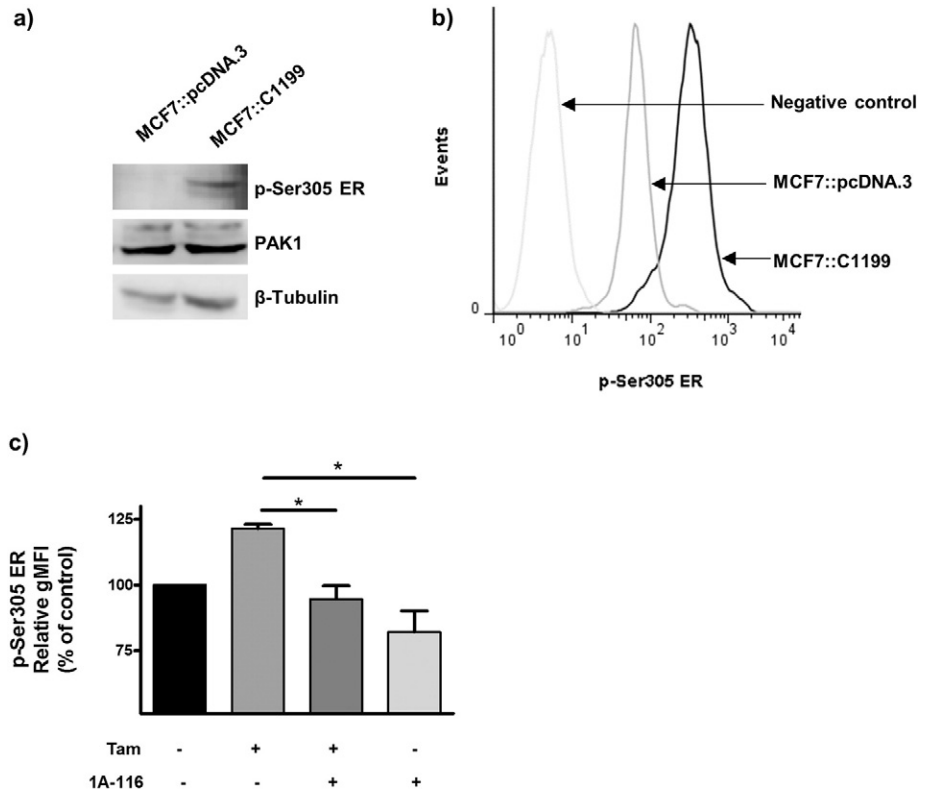

Fig. 5. 1A-116 decreased levels of phospho-Ser305 ER in response to Rac1 upregulation and Tam treatment. a) Cell lysates of MCF7::pcDNA.3 and MCF7::C1199 were separated by SDS-PAGE and phospho-Ser305 ER and PAK1 levels were detected by Western blot. b) Phospho-Ser305 ER levels were also detected by flow cytometry in both cell lines. c) MCF7::C1199 cells were treated with Tam (1 μM), 1A-116 (4 μM) or combination of both drugs for 24 h and phospho-Ser305 ER levels were detected by flow cytometry. Phospho-Ser305 ER relative geometric mean fluorescence intensity (gMFI) were calculated as described in Materials and Methods. Error bars, S.E.M. ANOVA, Tukey's Multiple Comparison Test, *$p < 0.05$ versus Tam (stimulation treatment).

5. Conclusions

- Upregulation of the Rac1-PAK1 pathway triggered a hormone-independent and Tam resistant phenotype.
- We demonstrated that this signaling pathway is strongly stimulated in response to Tam.
- Resistance mechanisms, such PAK1 nuclear translocation and phosphorylation at Ser305 of ER, were reverted by 1A-116, a Rac1 inhibitor developed by our group.
- Since ER phospho-Ser305 positive patients do not respond to Tam treatment, blocking this phosphorylation site by Rac1 inhibition may become a new approach to restore Tam sensibility in breast cancer patients with acquired endocrine resistance.

Conflict of interest

The authors declare no conflicts of interest.

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Authors contributions

GN, CGA, GDE and MPL conception and design of research; CMJ designed and synthetized 1A-116; GN, CGA, SVI performed experiments; GN, CGA, SVI, PM, GDE and MPL analyzed data and interpreted results; GN prepared figures; GN, CGA, GDE and MPL drafted, edited, revised and approved final version of manuscript. All authors read and approved the final manuscript.

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References

[1] J.S.I. Ferlay, M. Ervik, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet], 2012.
[2] M.J. Higgins, J. Baselga, Targeted therapies for breast cancer, J. Clin. Invest. 121 (10) (2011) 3797–3803, http://dx.doi.org/10.1172/JCI57152.
[3] M.J. Piccart-Gebhart, New developments in hormone receptor-positive disease, Oncologist 16 (Suppl. 1) (2011) 40–50, http://dx.doi.org/10.1634/theoncologist.2011-S1-40.
[4] E.A. Musgrove, R.L. Sutherland, Biological determinants of endocrine resistance in breast cancer, Nat. Rev. Cancer 9 (9) (2009) 631–643, http://dx.doi.org/10.1038/nrc2713.
[5] R. Viedma-Rodriguez, L. Baiza-Gutman, F. Salamanca-Gomez, M. Diaz-Zaragoza, G. Martinez-Hernandez, R. Ruiz Esparza-Garrido, M.A. Velazquez-Flores, D. Arenas-Aranda, Mechanisms associated with resistance to tamoxifen in estrogen receptor-positive breast cancer (review), Oncol. Rep. 32 (1) (2014) 3–15, http://dx.doi.org/10.3892/or.2014.3190.

[6] E. Paplomata, R. O'Regan, The PI3K/AKT/mTOR pathway in breast cancer: targets, trials and biomarkers, Ther. Adv. Med. Oncol. 6 (4) (2014) 154–166, http://dx.doi.org/10.1177/1758834014530023.

[7] E.E. Bosco, J.C. Mulloy, Y. Zheng, Rac1 GTPase: a "Rac" of all trades, Cell. Mol. Life Sci. 66 (3) (2009) 370–374, http://dx.doi.org/10.1007/s00018-008-8552-x.

[8] A.J. Cardozo, D.E. Gomez, P.F. Argibay, Neurogenic differentiation of human adipose-derived stem cells: relevance of different signaling molecules, transcription factors, and key marker genes, Gene 511 (2) (2012) 427–436, http://dx.doi.org/10.1016/j.gene.2012.09.038.

[9] S. Shahjouei, S. Ansari, T. Pourmotabbed, R. Zand, Potential roles of adropin in central nervous system: review of current literature, Front. Mol. Biosci. 3 (2016) 25, http://dx.doi.org/10.3389/fmolb.2016.00025.

[10] D. Cai, A. Iyer, K.N. Felekkis, R.I. Near, Z. Luo, J. Chernoff, C. Albanese, R.G. Pestell, A. Lerner, AND-34/BCAR3, a GDP exchange factor whose overexpression confers anti-estrogen resistance, activates Rac, PAK1, and the cyclin D1 promoter, Cancer Res. 63 (20) (2003) 6802–6808.

[11] A.E. Rosenblatt, M.I. Garcia, L. Lyons, Y. Xie, C. Maiorino, L. Desire, J. Slingerland, K.L. Burnstein, Inhibition of the Rho GTPase, Rac1, decreases estrogen receptor levels and is a novel therapeutic strategy in breast cancer, Endocr. Relat. Cancer 18 (2) (2011) 207–219, http://dx.doi.org/10.1677/ERC-10-0049.

[12] S. Balasenthil, C.J. Barnes, S.K. Rayala, R. Kumar, Estrogen receptor activation at serine 305 is sufficient to upregulate cyclin D1 in breast cancer cells, FEBS Lett. 567 (2–3) (2004) 243–247, http://dx.doi.org/10.1016/j.febslet.2004.04.071.

[13] J. Tao, P. Oladimeji, L. Rider, M. Diakonova, PAK1-Nck regulates cyclin D1 promoter activity in response to prolactin, Mol. Endocrinol. 25 (9) (2011) 1565–1578, http://dx.doi.org/10.1210/me.2011-0062.

[14] C. Holm, M. Kok, R. Michalides, R. Fles, R.H. Koornstra, J. Wesseling, M. Hauptmann, J. Neefjes, J.L. Peterse, O. Stal, G. Landberg, S.C. Linn, Phosphorylation of the oestrogen receptor alpha at serine 305 and prediction of tamoxifen resistance in breast cancer, J. Pathol. 217 (3) (2009) 372–379, http://dx.doi.org/10.1002/path.2455.

[15] C. Holm, S. Rayala, K. Jirstrom, O. Stal, R. Kumar, G. Landberg, Association between Pak1 expression and subcellular localization and tamoxifen resistance in breast cancer patients, J. Natl. Cancer Inst. 98 (10) (2006) 671–680, http://dx.doi.org/10.1093/jnci/djj185.

[16] J. Bostner, L. Skoog, T. Fornander, B. Nordenskjold, O. Stal, Estrogen receptor-alpha phosphorylation at serine 305, nuclear p21-activated kinase 1 expression, and response to tamoxifen in postmenopausal breast cancer, Clin. Cancer Res. 16 (5) (2010) 1624–1633, http://dx.doi.org/10.1158/1078-0432.CCR-09-1733.

[17] H. Aguilar, A. Urruticoechea, P. Halonen, K. Kiyotani, T. Mushiroda, X. Barril, J. Serra-Musach, A. Islam, L. Caizzi, L. Di Croce, E. Nevedomskaya, W. Zwart, J. Bostner, E. Karlsson, G. Perez Tenorio, T. Fornander, D.C. Sgroi, R. Garcia-Mata, M.P. Jansen, N. Garcia, N. Bonifaci, F. Climent, M.T. Soler, A. Rodriguez-Vida, M. Gil, J. Brunet, G. Martrat, L. Gomez-Baldo, A.I. Extremera, A. Figueras, J. Balart, R. Clarke, K.L. Burnstein, K.E. Carlson, J.A. Katzenellenbogen, M. Vizoso, M. Esteller, A. Villanueva, A.B. Rodriguez-Pena, X.R. Bustelo, Y. Nakamura, H. Zembutsu, O. Stal, R.L. Beijersbergen, M.A. Pujana, VAV3 mediates resistance to breast cancer endocrine therapy, Breast Cancer Res. 16 (3) (2014) R53, http://dx.doi.org/10.1186/bcr3664.

[18] G.A. Cardama, M.J. Comin, L. Hornos, N. Gonzalez, L. Defelipe, A.G. Turjanski, D.F. Alonso, D.E. Gomez, P.L. Menna, Preclinical development of novel Rac1-GEF signaling inhibitors using a rational design approach in highly aggressive breast cancer cell lines, Anti Cancer Agents Med. Chem. 14 (6) (2014) 840–851.

[19] G.A. Cardama, N. Gonzalez, M. Ciarlantini, L. Gandolfi Donadio, M.J. Comin, D.F. Alonso, P.L. Menna, D.E. Gomez, Proapoptotic and antiinvasive activity of Rac1 small molecule inhibitors on malignant glioma cells, Onco. Targets Ther. 7 (2014) 2021–2033, http://dx.doi.org/10.2147/OTT.S67998.

[20] J.C. Stam, E.E. Sander, F. Michiels, F.N. van Leeuwen, H.E. Kain, R.A. van der Kammen, J.G. Collard, Targeting of Tiam1 to the plasma membrane requires the cooperative function of the N-terminal pleckstrin homology domain and an adjacent protein interaction domain, J. Biol. Chem. 272 (45) (1997) 28447–28454.

[21] G.G. Habets, E.H. Scholtes, D. Zuydgeest, R.A. van der Kammen, J.C. Stam, A. Berns, J.G. Collard, Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins, Cell 77 (4) (1994) 537–549.

[22] P. Holden, W.A. Horton, Crude subcellular fractionation of cultured mammalian cell lines, BMC Res. Notes 2 (2009) 243, http://dx.doi.org/10.1186/1756-0500-2-243.

[23] S. Chow, H. Patel, D.W. Hedley, Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: potential for pharmacodynamic monitoring of signal transduction inhibitors, Cytometry 46 (2) (2001) 72–78.

[24] L. Adam, R.K. Vadlamudi, P. McCrea, R. Kumar, Tiam1 overexpression potentiates heregulin-induced lymphoid enhancer factor-1/beta -catenin nuclear signaling in breast cancer cells by modulating the intercellular stability, J. Biol. Chem. 276 (30) (2001) 28443–28450, http://dx.doi.org/10.1074/jbc.M009769200.

[25] T. Zhuang, J. Zhu, Z. Li, J. Lorent, C. Zhao, K. Dahlman-Wright, S. Stromblad, p21-activated kinase group II small compound inhibitor GNE-2861 perturbs estrogen receptor alpha signaling and restores tamoxifen-sensitivity in breast cancer cells, Oncotarget 6 (41) (2015) 43853–43868, http://dx.doi.org/10.18632/oncotarget.6081.

[26] R. Houtman, R. de Leeuw, M. Rondaij, D. Melchers, D. Verwoerd, R. Ruijtenbeek, J.W. Martens, J. Neefjes, R. Michalides, Serine-305 phosphorylation modulates estrogen receptor alpha binding to a coregulator peptide array, with potential application in predicting responses to tamoxifen, Mol. Cancer Ther. 11 (4) (2012) 805–816, http://dx.doi.org/10.1158/1535-7163.MCT-11-0855.

[27] P. Oladimeji, R. Skerl, C. Rusch, M. Diakonova, Synergistic activation of ERalpha by estrogen and prolactin in breast cancer cells requires tyrosyl phosphorylation of PAK1, Cancer Res. 76 (9) (2016) 2600–2611, http://dx.doi.org/10.1158/0008-5472.CAN-15-1758.

[28] M.J. Piccart-Gebhart, New developments in hormone receptor-positive disease, Oncologist 15 (Suppl. 5) (2010) 18–28, http://dx.doi.org/10.1634/theoncologist.2010-S5-18.

[29] A.J. Ellis, V.M. Hendrick, R. Williams, B.S. Komm, Selective estrogen receptor modulators in clinical practice: a safety overview, Expert Opin. Drug Saf. 14 (6) (2015) 921–934, http://dx.doi.org/10.1517/14740338.2015.1014799.

[30] A. Hackshaw, Luteinizing hormone-releasing hormone (LHRH) agonists in the treatment of breast cancer, Expert. Opin. Pharmacother. 10 (16) (2009) 2633–2639, http://dx.doi.org/10.1517/14656560903224980.

[31] N. Heldring, A. Pike, S. Andersson, J. Matthews, G. Cheng, J. Hartman, M. Tujague, A. Strom, E. Treuter, M. Warner, J.A. Gustafsson, Estrogen receptors: how do they signal and what are their targets, Physiol. Rev. 87 (3) (2007) 905–931, http://dx.doi.org/10.1152/physrev.00026.2006.

[32] B. Saville, M. Wormke, F. Wang, T. Nguyen, E. Enmark, G. Kuiper, J.A. Gustafsson, S. Safe, Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements, J. Biol. Chem. 275 (8) (2000) 5379–5387.

[33] L. Bjornstrom, M. Sjoberg, Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes, Mol. Endocrinol. 19 (4) (2005) 833–842, http://dx.doi.org/10.1210/me.2004-0486.

[34] R. Clarke, N. Brunner, B.S. Katzenellenbogen, E.W. Thompson, M.J. Norman, C. Koppi, S. Paik, M.E. Lippman, R.B. Dickson, Progression of human breast cancer cells from hormone-dependent to hormone-independent growth both in vitro and in vivo, Proc. Natl. Acad. Sci. U. S. A. 86 (10) (1989) 3649–3653.

[35] H. Marei, A. Malliri, Rac1 in Human Diseases: The Therapeutic Potential of Targeting Rac1 Signaling Regulatory Mechanisms. Small GTPases, 0, 2016http://dx.doi.org/10.1080/21541248.2016.1211398.

[36] E. Hodis, I.R. Watson, G.V. Kryukov, S.T. Arold, M. Imielinski, J.P. Theurillat, E. Nickerson, D. Auclair, L. Li, C. Place, D. Dicara, A.H. Ramos, M.S. Lawrence, K. Cibulskis, A. Sivachenko, D. Voet, G. Saksena, N. Stransky, R.C. Onofrio, W. Winckler, K. Ardlie, N. Wagle, J. Wargo, K. Chong, D.L. Morton, K. Stemke-Hale, G. Chen, M. Noble, M. Meyerson, J.E. Ladbury, M.A. Davies, J.E. Gershenwald, S.N. Wagner, D.S. Hoon, D. Schadendorf, E.S. Lander, S.B. Gabriel, G. Getz, L.A. Garraway, L. Chin, A landscape of driver mutations in melanoma, Cell 150 (2) (2012) 251–263, http://dx.doi.org/10.1016/j.cell.2012.06.024.

[37] M. Krauthammer, Y. Kong, B.H. Ha, P. Evans, A. Bacchiocchi, J.P. McCusker, E. Cheng, M.J. Davis, G. Goh, M. Choi, S. Ariyan, D. Narayan, K. Dutton-Regester, A. Capatana, E.C. Holman, M. Bosenberg, M. Sznol, H.M. Kluger, D.E. Brash, D.F. Stern, M.A. Materin, R.S. Lo, S. Mane, S. Ma, K.K. Kidd, N.K. Hayward, R.P. Lifton, J. Schlessinger, T.J. Boggon, R. Halaban, Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma, Nat. Genet. 44 (9) (2012) 1006–1014, http://dx.doi.org/10.1038/ng.2359.

[38] K.L. Rossman, C.J. Der, J. Sondek, GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors, Nat. Rev. Mol. Cell Biol. 6 (2) (2005) 167–180, http://dx.doi.org/10.1038/nrm1587.

[39] D. Joyce, B. Bouzahzah, M. Fu, C. Albanese, M. D'Amico, J. Steer, J.U. Klein, R.J. Lee, J.E. Segall, J.K. Westwick, C.J. Der, R.G. Pestell, Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor-kappaB-dependent pathway, J. Biol. Chem. 274 (36) (1999) 25245–25249.

[40] S. Hiscox, W.G. Jiang, K. Obermeier, K. Taylor, L. Morgan, R. Burmi, D. Barrow, R.I. Nicholson, Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of beta-catenin phosphorylation, Int. J. Cancer 118 (2) (2006) 290–301, http://dx.doi.org/10.1002/ijc.21355.

[41] S.K. Rayala, P.R. Molli, R. Kumar, Nuclear p21-activated kinase 1 in breast cancer packs off tamoxifen sensitivity, Cancer Res. 66 (12) (2006) 5985–5988, http://dx.doi.org/10.1158/0008-5472.CAN-06-0978.

[42] J. Bostner, M. Ahnstrom Waltersson, T. Fornander, L. Skoog, B. Nordenskjold, O. Stal, Amplification of CCND1 and PAK1 as predictors of recurrence and tamoxifen resistance in postmenopausal breast cancer, Oncogene 26 (49) (2007) 6997–7005, http://dx.doi.org/10.1038/sj.onc.1210506.

[43] P. Oladimeji, M. Diakonova, PAK1 translocates into nucleus in response to prolactin but not to estrogen, Biochem. Biophys. Res. Commun. 473 (1) (2016) 206–211, http://dx.doi.org/10.1016/j.bbrc.2016.03.079.

[44] A. Ghosh, S. Awasthi, J.R. Peterson, A.W. Hamburger, Regulation of tamoxifen sensitivity by a PAK1-EBP1 signalling pathway in breast cancer, Br. J. Cancer 108 (3) (2013) 557–563, http://dx.doi.org/10.1038/bjc.2013.11.

[45] R.A. Wang, A. Mazumdar, R.K. Vadlamudi, R. Kumar, P21-activated kinase-1 phosphorylates and transactivates estrogen receptor-alpha and promotes hyperplasia in mammary epithelium, EMBO J. 21 (20) (2002) 5437–5447.

[46] S.K. Rayala, A.H. Talukder, S. Balasenthil, R. Tharakan, C.J. Barnes, R.A. Wang, C.M. Aldaz, S. Khan, R. Kumar, P21-activated kinase 1 regulation of estrogen receptor-alpha activation involves serine 305 activation linked with serine 118 phosphorylation, Cancer Res. 66 (3) (2006) 1694–1701, http://dx.doi.org/10.1158/0008-5472.CAN-05-2922.

[47] R. de Leeuw, J. Neefjes, R. Michalides, A role for estrogen receptor phosphorylation in the resistance to tamoxifen, Int. J. Breast Cancer 2011 (2011) 232435, http://dx.doi.org/10.4061/2011/232435.

[48] L.C. Murphy, S.V. Seekallu, P.H. Watson, Clinical significance of estrogen receptor phosphorylation, Endocr. Relat. Cancer 18 (1) (2011) R1–14, http://dx.doi.org/10.1677/ERC-10-0070.

[49] R. Michalides, A. Griekspoor, A. Balkenende, D. Verwoerd, L. Janssen, K. Jalink, A. Floore, A. Velds, L. van't Veer, J. Neefjes, Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer, Cancer Cell 5 (6) (2004) 597–605, http://dx.doi.org/10.1016/j.ccr.2004.05.016.