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Progesterone Sensitizes Breast Cancer MCF7 Cells to Imatinib Inhibitory Effects

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Abstract In previous studies, we found that progesterone was able to induce the expression of platelet-derived growth factor (PDGF) in human breast cancer MCF7 cells. Knowing that imatinib mesylate targets PDGF receptor tyrosine kinase activity, the aim of the present study was to examine the effects of imatinib on progesterone-treated MCF7 cells. Expression of phosphorylated (activated) platelet-derived growth factor receptor-α (PDGFRα) was detected in MCF7 cells. Interestingly, phosphorylated-PDGFRα expression was significantly downregulated by imatinib. The effects of imatinib on cell growth, apoptosis and migration were then analyzed. Imatinib effectively inhibited anchorage-dependent colony formation, and cell viability as evaluated by MTT assay. Corroborating these findings, a significant increase in the percentage of apoptotic cells was also observed when cells were treated with imatinib. Surprisingly, these inhibitory effects were all enhanced by the presence of progesterone. Cell migration assays did also show a reduction in the migratory capacity after incubation with imatinib. These findings reveal that imatinib acts by decreasing MCF7 cell viability, growth and migration, with concomitant increase in apoptosis. Furthermore, incubation with progesterone seems to prompt cells to the inhibitory action of imatinib, probably by sustaining PDGFRα activity. The current study points out imatinib as a possible therapeutic strategy in progesterone-dependent breast cancer. J. Cell. Biochem. 103: 607–614, 2008.
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Key words: breast cancer; hormonal signaling; imatinib; PDGF; progesterone; tyrosine kinase

Breast cancer continues to be the most common cancer and the second leading cause of cancer death and morbidity among women in the western world [Soares et al., 2007]. Therefore, the constant search for new therapeutic targets which may result in better disease-free and overall survival becomes of primary relevance [Carvalho et al., 2005; Gonzales-Angulo et al., 2006].

Imatinib is a phenylaminopyrimidine analogue drug that competes with ATP for its specific binding site in the kinase domain of specific tyrosine kinase receptors such as c-Kit and the platelet-derived growth factor receptor (PDGFR) [Mauro and Druker, 2001; Buchdunger et al., 2002; Jones and Judson, 2005]. Accordingly, imatinib mesylate was first developed as an inhibitor of platelet-derived growth factor receptor-α (PDGFRα) tyrosine kinase activity. It is also able to inhibit Abl tyrosine kinase variants as well as c-kit, the receptor of stem cell factor (SCF). Therefore, as several studies report, imatinib has been successfully used to treat chronic myeloid leukemia (CML) [Radford, 2002; Mauro and Druker, 2001; Panigrahi and Naithani, 2006; Kantarjian and Cortes, 2006]. Clinical evidence indicates that imatinib also targets gastrointestinal stromal tumors (GIST), which are characterized by the presence of the cell surface transmembrane receptor c-kit [Tuveson et al., 2001; Radford, 2002]. GIST frequently present mutations in c-kit gene. This leads to constitutive activation of this membrane receptor, mediating uncontrolled cell growth and survival. Although changes in the PDGF signaling pathway have been largely described in breast cancer, the effect of imatinib in this type of tumors has only barely been examined [Roussidis et al., 2004; Modi et al., 2005]. PDGFRα is expressed in nearly 40% of invasive breast cancer, and correlates with poor prognostic

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parameters [Carvalho et al., 2005], what renders imatinib a potential therapeutic target against these tumors.

Previous studies of our group showed that incubation of hormone-dependent breast cancer MCF7 cells with progesterone (P4) results in the expression of several genes, namely those involved in platelet-derived growth factor (PDGF) signaling pathway [Soares et al., 2007]. PDGF receptors (PDGFRs) α e β are characterized by an intracellular tyrosine kinase domain and its action depends on ligand binding [George, 2001; Alvarez et al., 2006]. PDGFR signaling is involved in crucial roles within the cells, namely proliferation, survival, and maintenance of an invasive phenotype [Tallquist and Kazlauskas, 2004]. Furthermore, PDGFR overexpression is associated with several pathologies such as vasculoproliferative diseases and cancer [Ostman, 2004]. Taken together, all these facts prompted us to investigate whether PDGF inhibitory agent imatinib is a useful therapeutic drug in hormone-dependent cancer.

MATERIALS AND METHODS

Chemicals

Progesterone (P4) was obtained from Sigma–Aldrich (Portugal). Imatinib was kindly provided by Novartis. Antibodies against total-PDGFRα, phosphorylated-PDGFRα and βactin (Santa Cruz Biotechnology) were used for immunoblotting.

P4 was dissolved in 100% ethanol and added to serum-free medium of cell cultures at a final concentration of 10^{-8} M. Concentration for P4 was chosen from a set of five different concentrations ranging from 0.1 to 1,000 nM in previous studies [Soares et al., 2007]. Ethanol was added to cell culture media at a concentration of 0,01% (v/v). Imatinib was dissolved in distilled water and added to serum-free medium of cell cultures at a range of final concentrations of 1, 2.5, 5, 7.5, and 10 μM.

Because P4 receptor (PR) is an estrogen-dependent gene product, cells were maintained in 10% FBS and 1% penicillin/streptomycin until 70% confluence, then washed twice with PBS and immediately incubated in serum-free conditions, with no prior serum-free medium incubation, as previously described [Soares et al., 2007].

Cell Cultures

Human breast cancer cell line, MCF7, was obtained from the American Type Culture Collection (ATCC, Barcelona, Spain). MCF7 cells were routinely cultured in Eagle MEM medium containing 10% inactivated FBS and 1% penicillin/streptomycin.

Cell culture media, FBS and penicillin/streptomycin were purchased from Invitrogen Life Technologies (Paisley, Scotland, UK). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. In agreement with previous studies, incubations with the distinct agents were performed for 24 h in serum-free conditions [Soares et al., 2007].

Clonogenic Assay

Colony formation assay was performed as previously described [Mittal et al., 2004]. Briefly, cells were harvested at a concentration of 1×10^3 cells/plate in 21 cm plates, incubated for 24 h in complete medium and then incubated with ethanol (EtOH), 10^{-8} M P4, or combinations of EtOH or P4 with imatinib for 7 days, enabling each cell to proliferate and form colonies. Cell cultures were then washed with PBS, fixed with 70% ethanol and stained with 0.04% trypan blue solution. Colonies with more than 20 cells were counted on a phase contrast microscope (Nikon, UK). Results were expressed in percentage of the colonies formed by the control cells (ethanol treated).

MTT Proliferation Assay

MCF7 cells were cultured following standard conditions or the treatment procedures for 24 h. Cells were then washed twice with PBS and subjected to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, an index of cell viability and cell growth. Cells were incubated with MTT solution at a final concentration of 0.5 mg/ml for 3 h and then lysed in DMSO. Optical density was measured at 540 nm. The background absorbance of the medium in the absence of cells was subtracted. All samples were assayed in triplicate, and the mean value for each experiment was calculated. The results are given as mean (SD) and are expressed as percentage of control, which was considered to be 100%.

Western Blotting

Proteins were isolated from MCF7 cell lysates using Tripure (Roche Diagnostics, Basel, Switzerland). Secreted proteins were precipitated from cell medium with absolute ethanol at −20°C. Proteins were quantified using a

spectrophotometer (Jenway, 6405 UV/vis, Essex, UK) and equal amounts of protein were subjected to 8% or 15% SDS–PAGE with a 5% stacking gel. After electrophoresis proteins were blotted into a Hybond nitrocellulose membrane (Amersham, Arlington), using a mini-transblot electrophoretic transfer cell (Amersham Biosciences). Immunodetection for total PDGFRα, phosphorylated (activated) PDGFRα (Pi-PDGFRα) and βactin was accomplished with enhanced chemiluminiscence (ECL kit, Amersham Biosciences). The relative intensity of each protein blotting analysis was measured using a computerized software program (Biorad, Portugal) and normalized with βactin bands to compare the expression of proteins in different treatment groups. Experiments were repeated three times.

TUNEL Assays

Cells grown until 70% confluence onto glass coverslips were incubated with the different referred treatments for 24 h. TUNEL assay (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) was performed using the In Situ Cell Death Detection kit (Roche Diagnostics), according to the manufacturer's instructions. Nuclei were counter-stained with DAPI (Roche Diagnostics). The percentage of TUNEL-stained nuclei was evaluated in relation to every DAPI-stained nucleus observed. Immunofluorescence was visualized under a fluorescence microscope (Olympus, BH-2, UK). The percentage of stained cells was evaluated by counting the cells stained with TUNEL divided by the total number of nuclei stained with DAPI at a 200× magnification field. One thousand nuclei were evaluated. Three independent experiments were performed.

BrdU Incorporation Assay

MCF7 cells (1×10^4) grown on coverslips were incubated with different concentrations of imatinib (1, 2.5, 5, 7.5, or 10 μM) in serum-free medium for 24 h. Control cells (0) were incubated in serum-free medium. Every cell culture was also incubated with Bromodeoxyuridine (BrdU), which incorporates into DNA in dividing cells only. BrdU-stained cells were then evaluated by immunohistochemistry using a commercially available BrdU incorporation assay kit (BD-Biosciences, Belgium) according to the manufacturer's instructions. BrdU immunostaining was then visualized on an optical microscope (Nikon, UK) at a magnification of 200×. The percentage of stained nuclei was evaluated on a total of 1,000 cells.

Migration Analysis

Injury assay. Injury assay was performed as previously described [Soares et al., 2007]. Cells were grown to 90% confluence. Using a pipette tip, cells were scrapped from the culture dish leaving a void space. Cells were then incubated for 24 h following the standard treatments. After incubation cells were washed with PBS and cell migration to the damaged area was visualized and photographed on a phase contrast microscope (Nikon, UK). Magnification 200×.

Double-chamber assay. The migration capacity of MCF7 cells was then quantified by counting the number of cells that migrated through matrigel-coated transwell BD-matrigel basement membrane matrix inserts (BD-Biosciences). Transwell inserts containing an 8 μm pore-size PET membrane coated with a uniform layer of Matrigel basement membrane were used. MCF7 cells (5×10^4) were harvested on inserts in serum-free medium, and placed on wells containing Eagle MEM medium complemented with FBS (10%), penicillin/streptomycin (1%) and EtOH, P4 or a combination of EtOH or P4 with imatinib. After incubation for 24 h at 37°C and 5% CO_2, membranes were removed from inserts, stained with DAPI-methanol (Roche Diagnostics) for 5 min and visualized under a fluorescence microscope (Olympus, BH-2). Twenty-five random fields of each membrane were counted on the microscope (200×).

Statistical Analyses

All experiments were performed in triplicate. Quantifications are expressed in mean (SD). Samples were evaluated by the analysis of variance test. A difference between experimental groups was analyzed by Student's t-test, and was considered statistically significant whenever the P-value was less than 5%.

RESULTS

Imatinib Reduced MCF7 Cell Proliferation and Increased Apoptosis in a Dose-Dependent Manner

We first determined the dose-response effect of imatinib on cell apoptosis and proliferation by

TUNEL and BrdU incorporation assays respectively. Treatment of MCF7 cells with 1–10 μM imatinib for 24 h resulted in a dose-dependent increase in the percentage of apoptotic cells (Fig. 1A). This increase was statistically significant for 5 μM or higher. The percentage of proliferative cells also decreased in a dose-dependent manner, reaching significant reduction for concentration of equal or higher than 2.5 μM. According to these findings and in agreement with the literature [Roussidis et al., 2004], imatinib was used at a concentration of 5 μM in subsequent studies.

Presence of PDGF Receptor in MCF7 Cells

To ensure that imatinib could be effective in MCF7 cells, we then confirmed the expression of PDGF receptors (PDGFR) in MCF7 cells by Western blotting. The expression of total PDGFRα was observed in MCF7 cell lysates after each treatment. However, in the presence of imatinib both total and phosphorylated PDGFRα were downregulated. This reduction in the expression and activation of PDGFRα was more evident in the presence of P4 (Fig. 2).

Imatinib Decreased Cell Viability, Proliferation, and Increased Apoptosis

To determine the effect of imatinib on breast cancer MCF7 cells behavior, the anchorage-dependent colony formation assay was used. In comparison to ethanol, treatment of MCF7 cells with P4 resulted in an increase in colony formation potential (Fig. 3A). Incubation of cells with imatinib either in the presence or in the absence of P4 led to a significant reduction in the number of colonies formed (Figs. 3A).

The effect of imatinib in cell viability was further investigated by MTT assay. Incubation of MCF7 cells with P4 did not significantly affect cell viability in comparison to controls (Fig. 3B). A slight decrease in cell viability was found upon exposure to imatinib in the presence of vehicle (EtOH + imat), though not reaching statistical significance. However, treatment with imatinib together with P4 resulted in a significant decrease in MCF7 cells viability (Fig. 3B; $^{\#}P < 0.01$).

We then addressed the role of imatinib in the apoptotic levels of MCF7 cells using TUNEL

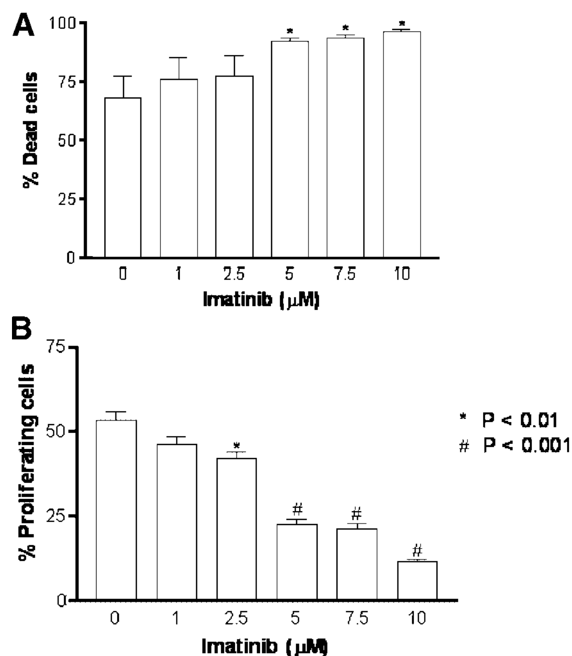

Fig. 1. Dose response of imatinib in MCF7 cells. Incubation of MCF7 cells with imatinib increased apoptosis and decreased proliferation in a dose-dependent manner. **A**: Percentage of apoptotic cells assessed by TUNEL assay. A significant increase in cell apoptosis relative to controls (0) was found whenever cells were incubated with 5, 7.5, and 10 μM imatinib ($^{*}P < 0.01$ vs. control). Cell apoptosis is reported as mean (SD). **B**: Percentage of proliferating cells assessed by BrdU incorporation assay. The percentage of BrdU staining cells was downregulated by imatinib in a dose-dependent manner, reaching statistical significant levels upon incubation with 2.5 μM ($^{*}P < 0.01$ vs. control) or higher ($^{\#}P < 0.001$ vs. control). Cell proliferation is reported as mean (SD).

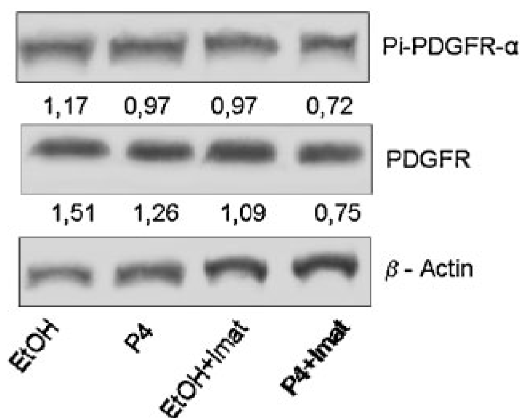

Fig. 2. Expression of total PDGFRα and activated (phosphorylated)-PDGFRα (Pi-PDGFRα) in MCF7 cell lysates after incubation with ethanol (EtOH), progesterone (P4), ethanol + imatinib (EtOH + Imat) or progesterone + imatinib (P4 + Imat) during 24 h. PDGFRα expression was found in every cell lysate. Pi-PDGFRα was slightly decreased in imatinib-treated cultures as compared to controls. The relative intensity of each band after normalization to βActin is shown under each blot, expressed as mean values. A representative Western blotting is shown from three independent experiments.

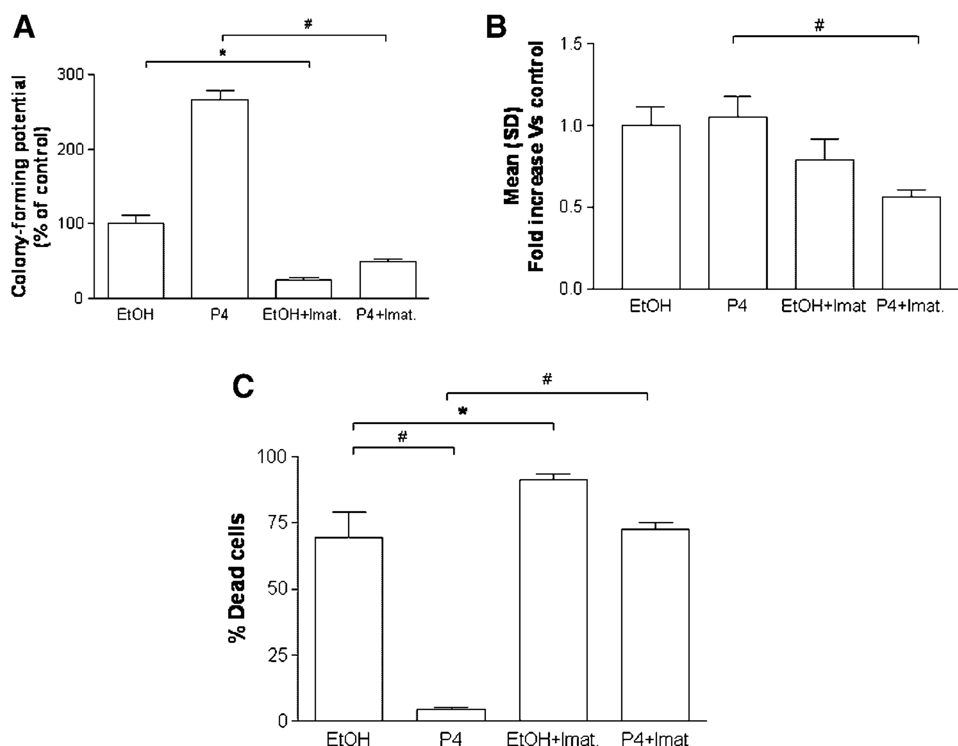

Fig. 3. In vitro treatment of human MCF7 breast cancer cells with imatinib decreased cell proliferation, cell viability, and increased apoptosis. **A**: Cell proliferation assessed using the clonogenic assay in MCF7 cells. Treatment of cells with 10^{-8} M P4 increased colony-forming potential recorded after 7-day treatment. Incubation of MCF7 cells with imatinib in the absence or in the presence of P4 (EtOH + Imat or P4 + Imat) resulted in a significant reduction in the number of colonies formation (*$P < 0.05$; #$P < 0.01$). Experiments were repeated three times with identical results. **B**: MCF7 cells viability by MTT assay. A slight increase in cell viability was found after incubation with P4 for 24 h. Incubation with imatinib led to a decrease in cell viability, being this decrease significant whenever cells were treated with imatinib combined with P4 (P4 + Imat) (#$P < 0.05$ vs. control). Cell viability is expressed in terms of percentage of control cells (EtOH), and is reported as mean (SD). **C**: Apoptosis was evaluated in MCF7 cells by TUNEL assay. P4 resulted in a strong reduction in the percentage of apoptotic cells (#$P < 0.01$ vs. EtOH). Treatment with imatinib significantly increased apoptosis (*$P < 0.05$). Imatinib significantly reversed the effect of P4 (*$P < 0.01$ vs. P4 alone). Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. Experiments were repeated three times with identical results.

analysis. As illustrated in Figure 3C, apoptosis was strongly reduced whenever cells were incubated with P4 (Fig. 3C). The presence of imatinib either alone or in combination with P4 potently increased the percentage of apoptotic cells (Fig. 3C; *$P < 0.05$ vs. control). No significant difference was found between P4 + imatinib and ethanol. Interestingly, imatinib completely reversed the survival effects of P4, leading to a statistically significant increase in the percentage of dead cells (#$P < 0.01$ vs. P4).

Imatinib Prevented Migration of MCF7 Cells

The migration ability was first examined using injury assay. Incubation with ethanol (EtOH) for 24 h resulted in the migration of a few cells into the damaged areas of cell culture (Fig. 4A). Migration capacity was intense upon cell incubation with P4, practically covering the whole injured area. Incubation with imatinib either in the presence or in the absence of P4 resulted in much less migration ability to the injured areas as compared to EtOH-treated and to P4-treated cell cultures (Fig. 4A). Remarkably, in contrast to imatinib in the presence of vehicle only (EtOH + Imat), the presence of imatinib combined with P4 resulted in an increase in the number of dead cells, as observed in Figure 4A (P4 + Imat).

To confirm that these findings were due to inhibition of cell migration by imatinib, and not just caused by increased apoptosis, the number of migrating cells was then quantified by double-chamber assay. In comparison to ethanol, P4 led to a strong increase in migration capacity of MCF7 cells (Fig. 4B). A significant decrease in the number of migrating cells was

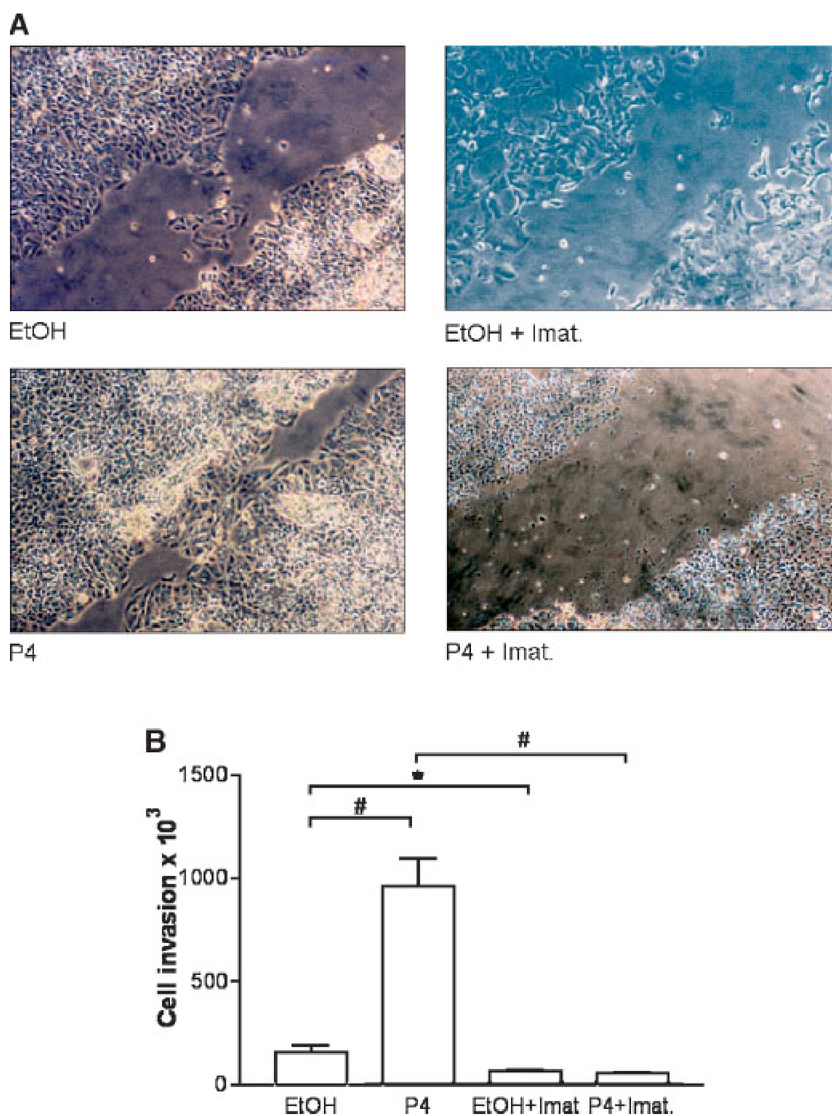

Fig. 4. Effects of imatinib in MCF7 cell migration. **A**: Cell migration was visualized by injury assay after incubation during 24 h. P4-treated cells rapidly migrated to the damaged area in comparison to control cells (ethanol-treated cells). Migration was not observed in cultures incubated with imatinib, either in the absence (EtOH + Imat) or in the presence of P4 (P4 + Imat). Note the increased number of dead cells whenever MCF7 cells were incubated with P4 + Imat. Pictures are representative of three independent studies. Magnification (200×). **B**: Migration was quantified in a double-chamber assay using medium complemented with 10% FBS as a chemoattractant. Incubation with P4 resulted in effective migration of cells ($^{\#}P < 0.01$ vs. EtOH). Treatment with imatinib completely abrogated migration capacity ($^{*}P < 0.05$ vs. EtOH and $^{\#}P < 0.01$ vs. P4). Bars represent the number of invasive cells. Assays were repeated twice and performed in triplicate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

found after incubation with imatinib independently of the presence of P4 (Fig. 4B).

DISCUSSION

The knowledge that breast tumors express PDGF has far been reported [Roussidis et al., 2004; Carvalho et al., 2005; Modi et al., 2005]. Nevertheless, only a few studies focused on the presence of PDGFR in the tumor cell membrane [Carvalho et al., 2005; Jechlinger et al., 2006]. In previous studies, our group has demonstrated that P4 resulted in increased expression of genes from the PDGF signaling pathway in MCF7 cells [Soares et al., 2007]. In addition, incubation of these cells with P4 led to increased cell growth and migration ability. These findings were reversed by incubation of cells with PDGF neutralizing antibodies, implying that P4-induced survival and proliferation was

accomplished by the interaction with PDGF signaling. The present study focused on the activity of imatinib, an inhibitor of PDGFRα tyrosine kinase activity, on the behavior of breast cancer MCF7 cells. We first showed that imatinib led to dose-dependent decreased cell proliferation and increased apoptosis. We also found out that total and active (phosphorylated) PDGFRα were reduced by imatinib, mainly in the presence of P4. These findings indicate that imatinib is able to both downregulate the expression and prevent phosphorylation of PDGFRα. These findings prompted us to examine whether imatinib could exert any effect in the behavior of MCF7 cells.

Moreover, we found that P4 induced proliferation, as regarded by the enlarged number of colonies formed during the 7-day period incubation. In contrast, incubation of cells with imatinib effectively prevented colony formation. This data was corroborated by the MTT viability assays. Accordingly, P4-treated cells presented slightly higher number of viable cells than controls (vehicle-treated cells). Incubation with imatinib efficiently decreased cell viability. In addition, this effect was only significant in the presence of P4. We, therefore, hypothesize that by inducing the activation of PDGFRα as previously demonstrated [Soares et al., 2007], P4 sensitizes tumor cells against imatinib action. Conversely, in the absence of P4, MCF7 cells are likely promoting other signaling pathways that result in increased proliferation and survival. The effect of P4 on MCF7 cell survival was also denoted by the marked reduction in apoptotic rates. This is not unexpected since P4 has been mainly regarded as a survival factor [Faivre and Lange, 2006; Soares et al., 2007]. Incubation with imatinib completely abrogated this effect, resulting in significantly increased percentage of apoptotic cells. Imatinib alone also resulted in increased number of apoptotic cells in comparison to ethanol-treated cells. Together with the findings on cell viability, this data emphasize that imatinib might be of primordial relevance in hormone-dependent breast cancer.

In agreement with the current data, a recent study by Roussidis et al., applying imatinib in breast cancer cell lines, showed that this agent significantly inhibited MCF7 proliferation, by maintaining cells at the G2/M cell cycle phase, which resulted in a concomitant decrease in S-phase. Conversely, they were not able to find an increase in apoptosis in any of the breast cancer cell lines studied upon incubation with imatinib. The current study focused on the role of imatinib in P4 pathway. Our results clearly showed a significant increase in apoptosis after imatinib incubation together with P4. However and in agreement with Roussidis et al. [2004], the increase found when imatinib was incubated with ethanol was much lower, emphasizing again the role of P4 in enhancing imatinib effects.

A Phase II clinical trial showed that imatinib resulted in significant toxicity in patients presenting metastatic breast cancer [Modi et al., 2005]. This trial was conducted on progressive metastatic breast cancer independently of prior therapeutic strategies. Despite the findings by Modi et al. [2005] are disappointing, these authors did definitely not target the cases having PDGF signaling activation. Accordingly, only 4 cases out of 13 presented kit or PDGFR expression. Given the activation of PDGF signaling pathway by progesterone, the current paper provides new data regarding the use of imatinib as beneficial against a particular type of breast cancer, which is progesterone-depend.

The current study also addressed the effect of imatinib on cell migration. As previously described [Soares et al., 2007], P4 strongly induced MCF7 migration. Inversely, imatinib completely reversed this effect. In accordance, the number of migrating cells strongly declined after incubation with imatinib independently of the presence or absence of P4. Although these findings encourage further studies, they already suggest that other signaling pathways might also be involved in cell migration ability, which are not stimulated by P4.

In conclusion, the current study focused on the effect of imatinib in breast cancer that responds to progesterone. Altogether, our findings showed that imatinib prevented cell viability, proliferation and migration, and increased apoptosis. Furthermore, these effects were enhanced by the concomitant incubation of cells with P4, implying that this hormone is rendering tumor cells prone to the action of imatinib. To our knowledge, the studies using imatinib mesylate in breast cancer clinical settings are rare. Only one study reported a phase II trial of imatinib monotherapy in metastatic breast cancer with ineffective results due to increased toxicity [Modi et al., 2005]. Although careful trials are needed in

order to test imatinib for safety and efficacy in progesterone-dependent breast cancer, the present data points to the successful use of imatinib as a therapeutic strategy in specific types of breast tumors, namely the ones depending on progesterone signaling pathways.

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