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ORIGINAL ARTICLE

Targeting c-Src kinase enhances tamoxifen's inhibitory effect on cell growth by modulating expression of cell cycle and survival proteins

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Abstract
Purpose Many studies have implicated the non-receptor tyrosine kinase c-Src in the development and metastatic progression of many types of cancer. In breast cancer, c-Src has been proposed to mediate the actions of estrogen in cell cycle progression.
Methods In this study we investigated the interaction between c-Src inhibition and estrogen receptor (ER) function using the ER-positive and tamoxifen-sensitive MCF-7 breast cancer cells.
Results Pharmacological inhibition of c-Src blocked estrogen-dependent proliferation in MCF-7 cells and enhanced the inhibitory effects of tamoxifen or estrogen-deprivation on cell growth. Maximum inhibition (95%) of cell growth was obtained when tamoxifen and c-Src blockade were combined. Inhibition of c-Src kinase decreased levels of the ER targets c-Myc and cyclin D1 expression but not of Bcl-2. Nevertheless, blocking c-Src kinase in tamoxifen-treated MCF-7 cells led to apoptosis. Inhibition of c-Src kinase altered the ratio of Mcl-1 isoforms in favor of cell death whereas expression of the proapoptotic molecules Bad, Bak, and Bax was not altered. Surprisingly, blocking ER function increased the levels of Bad phosphorylation at serine 112 (BadpS112), an inactive (nonapoptotic) form of Bad. This inactivation of Bad upon ER blockade seemed to depend on c-Src function as chemical inhibition of c-Src kinase reduced BadpS112 levels in cells with impaired ER function but not in estrogen-treated cells.
Conclusion These results indicate a crucial role for c-Src kinase in the survival of ER-positive breast cancer cells only when ER function is blocked. Therefore, this study suggests that targeting simultaneously c-Src and ER may effectively inhibit growth of ER-positive breast cancer.

Keywords Breast cancer · Estrogen receptor · c-Src tyrosine kinase · Cyclin D1 · c-Myc · Bad · Mcl-1

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Introduction

Estrogen plays important roles in breast cancer development and growth. This fact has led to the development of specific hormonal therapies to block estrogen receptor (ER) function, such as tamoxifen or to block estrogen production as with the use of aromatase inhibitors [1]. Unfortunately, after a certain period of response most tumors acquire resistance to hormonal therapy [2, 3]. To treat and prevent progression of ER-positive breast cancer, we need to understand more clearly the molecular pathways that allow tumors to become resistant to hormonal therapies. Blocking these pathways with novel therapies may prevent breast cancer growth and progression.

Recent studies have suggested a role for the non-receptor tyrosine kinase c-Src in tamoxifen resistance [4–6]. This tyrosine kinase has been implicated in the development and progression of many types of cancer including breast cancer [7–12]. Two independent studies have shown that ER-positive breast cancer cells that acquired resistance to tamoxifen overexpress the

active form of c-Src whereas tamoxifen-sensitive cells do not [4, 5]. Moreover, other studies have demonstrated that tamoxifen effects on ER can be regulated by c-Src or v-Src [13, 14]. The important role of c-Src in breast cancer progression is supported by overexpression of activated c-Src in metastatic breast cancer [5]. Collectively, these data suggest that activation of c-Src promotes breast cancer progression and represents a therapeutic target in the treatment of ER-positive breast cancer.

In addition to c-Src's role in acquisition of tamoxifen resistance, c-Src has been proposed to mediate ER-dependent proliferation. Estrogen can rapidly induce c-Src activation in breast cancer cells and cells transfected with ER [15, 16]. Activation of c-Src by estrogen is crucial for estrogen-stimulated cell cycle progression since inhibiting c-Src family kinases blocks estrogen-dependent proliferation [10, 15, 17]. Thus, these studies indicated that blocking c-Src may mimic the effect of hormonal therapy in cell proliferation. However, the role of c-Src in survival of ER-positive breast cancer cells is not well understood. Currently, it is not known whether blocking c-Src function in ER-positive breast cancer cells can enhance the growth inhibitory effects of hormonal therapies in ER-positive cells.

In this study we investigated the interaction between hormonal therapies and inhibition of c-Src family kinases. We evaluated whether pharmacological inhibition of c-Src family tyrosine kinases can cooperate with tamoxifen or estrogen-deprivation in blocking growth of ER-positive breast cancer cells. We report here that blocking ER and c-Src function simultaneously provides a greater growth inhibitory effect than blocking each pathway independently. We show evidence that c-Src kinase is crucial for survival of ER-positive breast cancer cells when ER function is blocked. Therefore, our results support the use of combined ER and c-Src blockade to treat ER-positive breast cancer.

Materials and methods

Antibodies and reagents

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) except for PP2, an inhibitor for c-Src family tyrosine kinases obtained from Tocris (Ellisville, MO, USA). Antibodies against pBad-S112, Bad, p-c-Src-Y416, and p-Paxillin-Y118 were obtained from Cell Signaling Technology (Beverly, MA, USA); Bax, Bcl-2, Bcl-x, and Paxillin from Neomarkers (Fremont, CA, USA); poly (ADP-ribose) polymerase (PARP) from BD Biosciences (Mountain View, CA, USA); cyclin D1 (DCS-6), c-Myc (9E10), Mcl-1, c-Src (B-12), and Bak were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and β-actin from Sigma.

Cell culture and growth assays

MCF-7 cells were maintained in DME media containing 5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). Cells were plated in six-wells at a density of 80,000 cells per well in maintenance media (5% FBS). At the start of the experiment cells were changed to phenol-red free DME supplemented with 5% charcoal/dextran stripped FBS (CSS) (Hyclone, Logan, UT, USA) alone or containing either 17β-estradiol (5 nM), or tamoxifen (0.5 or 1 μM).

All growth experiments were done using triplicate wells. For cell growth, the sulforhodamine (SRB) assay was performed and optical density measured at 570 nm [18]. This assay quantifies cell growth by measuring total protein content as a surrogate for cell growth. To ensure estrogen deprivation, cell growth was evaluated at day 7, except when indicated otherwise. To assess cell cycle progression, thymidine incorporation was performed after 24 h of treatment [19]. To determine whether tamoxifen enhanced the effect of inhibition of c-Src, values obtained at each dose of PP2 in the absence of tamoxifen were compared to those observed in the presence of tamoxifen using cells in CSS that received neither tamoxifen nor PP2 as controls.

Preparation of cell extracts and Western blot analysis

Cell lysates were prepared using an NP-40 buffer supplemented with a cocktail of protease and phosphatase inhibitors as previously described [19]. Equal amounts of protein were run on SDS-PAGE gradient gels (Invitrogen) and transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA). Incubation with primary antibodies was done for 3 h at room temperature or overnight at 4°C followed by incubation with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). For quantification of immunoblots, images were analyzed with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoprecipitation

After treatment of cells with different doses of PP2 (0–10 μM) for 6 h, cell extracts were prepared using M-Per (Pierce, Rockford, IL, USA) with a cocktail of protease and phosphatase inhibitors. Equal amounts of

protein (500–2,000 μg) were immunoprecipitated with a monoclonal antibody (mAb327) specific for c-Src/v-Src from Calbiochem (La Jolla, CA, USA). Immunoprecipitations were conducted for 3 h at 4°C. Immunoprecipitates were washed 2–3 times with NP-40 buffer [19] and processed for Western to determine the amount of c-SrcpY419 and total c-Src present.

Immunofluorescence

Cells were fixed in cold methanol for 5 min followed by permeabilization with acetone for 2 min. Incubation with mouse monoclonal antibody against Bax was performed for 1 h at 37°C followed by Cy3-conjugated antimouse antibody (Jakson ImmunoResearch Laboratories Inc., West Grove, PA, USA) using similar conditions. To stain DNA, DAPI (4′, 6-diamidino-2-phenylindole) was used at a concentration of 2 μg/ml. Micrographs were taken with IPLab software using a Nikon 300 inverted microscope.

Statistical analyses

Data values are mean ± standard deviation of triplicate measurements from epresentative experiments. To determine whether the changes in values in the presence or absence of tamoxifen at each dose of PP2 were significantly different the Student's *T*-test was used.

Results

Inhibition of c-Src activity decreases growth of MCF-7 cells

Several studies support a crucial role of c-Src in mediating estrogen's actions on cell proliferation and tamoxifen-resistant growth [5, 10, 15, 17]. In this study, we investigated whether inhibition of c-Src family tyrosine kinases with PP2, a commercially available inhibitor, can enhance the effect of estrogen-deprivation or tamoxifen treatment in tamoxifen-sensitive MCF-7 cells. In the initial experiments, to assure effective inhibition of c-Src in serum-containing media but avoid potential off-target effects we used PP2 at the dose of 10 μM [5, 20]. This dose is lower than the dose commonly used by others (20 or 30 μM PP2) to block active c-Src in ER-positive cancer cells such as MCF-7 cells [21–23]. To evaluate the effect of c-Src inhibition, we compared growth curves of MCF-7 cells in media containing 5% CSS alone (estrogen-deprivation) or with either 5 nM 17β-estradiol (E_2) or 1 μM tamoxifen in the presence or absence of PP2. As expected, estrogen promoted a time-dependent increase in cell growth reaching plateau (~fivefold) at day 12 (Fig. 1) while estrogen-deprivation or tamoxifen treatment prevented MCF-7 cell growth. Inhibition of c-Src efficiently blocked estrogen-stimulated proliferation as judged by the lack of increase in cell growth throughout the growth curve. When compare to estrogen-treated cells at day 7, targeting c-Src kinase inhibited cell growth by $73.84 \pm 12.24\%$ ($n = 6$). Combining c-Src blockade and estrogen-deprivation also efficiently inhibited ($84.63 \pm 5.44\%$, $n = 6$) proliferation of MCF-7 cells. However, maximum growth inhibition ($95.01 \pm 1.62\%$, $n = 6$) was observed when tamoxifen was combined with the c-Src inhibitor PP2. Thus, combined with c-Src blockade, addition of tamoxifen provided a statistically significant ($P = 0.00118$) decrease in cell proliferation compared to CSS alone (estrogen-deprivation). These results indicate that PP2 is effective in blocking estrogen-stimulated proliferation and, moreover, enhancing the inhibitory effect of tamoxifen or estrogen-deprivation. The addition of PP2 to hormonal therapies not only inhibited cell proliferation but, after several days of treatment, also decreased overall cell growth when compared to the starting point of the experiment (T = day 0) suggestive of cell death. Thus, combination of hormonal therapies and c-Src inhibition may lead to cell death. The decrease in overall cell growth, when ER and c-Src function are blocked simultaneously, suggests that c-Src is critical for the survival of ER-positive breast cancer cells in the absence of ER function.

To delineate further the effect of targeting c-Src family kinases and ER function in MCF-7 cell growth, we measured the dose-dependent effects of PP2 during estrogen-deprivation or tamoxifen treatment. As tamoxifen can have ER-independent effects at the micromolar range [24], we lowered the concentration of tamoxifen to 0.5 μM to avoid these confounding effects of tamoxifen. To assure PP2 was effectively blocking c-Src function in MCF-7 cells, we determined the dose-dependent effect of PP2 on tyrosine phosphorylation of Paxillin, a well known c-Src substrate [22, 25]. Treatment of MCF-7 cells with increasing amounts of PP2 for 6 h produce a dose-dependent decrease in Paxillin phosphorylation on tyrosine 118 (PaxpY118) (Fig. 2a) whether the cells were simultaneously treated with 0.5 μM tamoxifen or with 5 nM E_2. Immunoprecipitation of c-Src (Fig. 2b) also showed a dose-dependent decrease on the levels of autophosphorylated c-Src (c-SrcpY419). Thus, PP2 modulates activity of endogenous c-Src in a dose-dependent manner. Figure 2c shows the dose-dependent

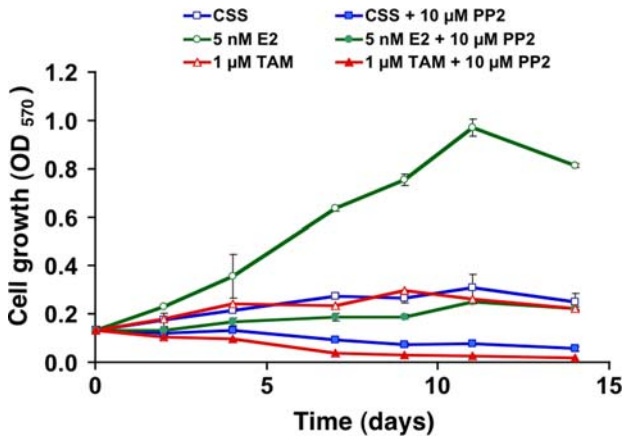

Fig. 1 Effect of inhibition of c-Src family tyrosine kinases on proliferation of MCF-7 cells. MCF-7 cells were grown in the presence of 5% CSS alone or supplemented with 5 nM 17β-estradiol or 1 μM tamoxifen in the presence or absence of the c-Src family tyrosine kinase inhibitor PP2. Cells were harvested at the times indicated and cell growth was measured by the SRB assay. The results are representative of at least three independent growth curves. *Values* represent the average ± standard deviation (SD) of triplicate wells from a representative experiment. In many time-points SD-values are within symbols

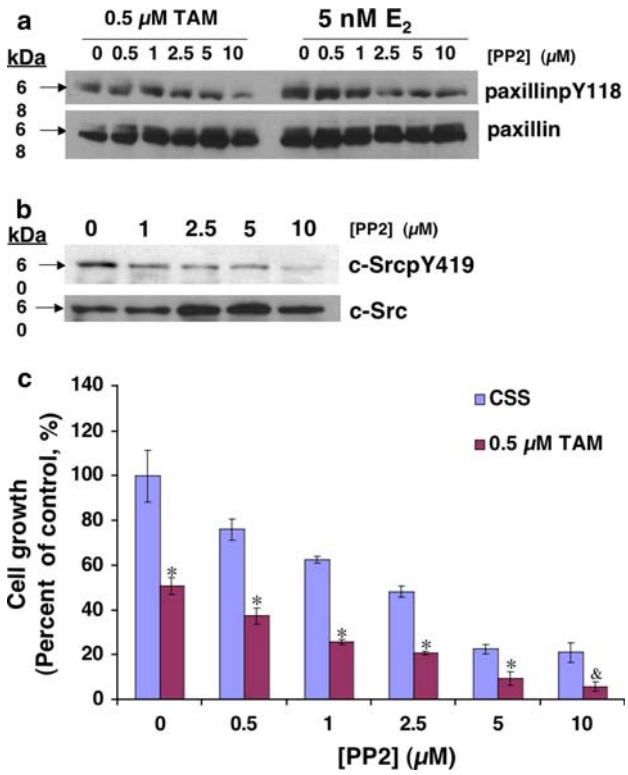

Fig. 2 Dose-dependent effect of PP2 on MCF-7 cells. **a** Dose-dependent effect of PP2 in tyrosine phosphorylation (PaxpY118) of the c-Src substrate Paxillin on MCF-7 cells treated as indicated for 6 h in media containing 5% CSS. **b** Immunoprecipitation of c-Src from MCF-7 cells treated for 6 h with different doses of PP2 in media containing 5% CSS and 0.5 μM Tamoxifen. **c** MCF-7 cells were cultured for 7 days in 5% CSS alone or in the presence of 0.5 μM tamoxifen with the indicated doses of PP2. Cell growth was measured using SRB. The results are representative of five independent experiments. *$P < 0.005$, & $P < 0.01$

effect of PP2 on MCF-7 cell growth in the presence or absence of tamoxifen. Even at the low dose of 0.5 μM PP2, as judged by its minor effect on PaxpY118, the combination of tamoxifen and PP2 produced a greater inhibition of cell growth compared to cells treated with the c-Src inhibitor and estrogen-deprivation. Nevertheless, maximum growth inhibition occurred when tamoxifen was combined with 5 or 10 μM PP2. As 5 μM PP2 gave comparable results to 10 μM we used 5 μM PP2 for all subsequent experiments. Therefore, these results suggest that blocking ER function and c-Src cooperate in decreasing growth of ER-positive breast cancer cells.

Effect of tamoxifen and inhibition of c-Src family kinases in cell cycle progression

Estrogen-dependent cell cycle progression depends on c-Src activation by estrogen [15, 17]. To evaluate the effect of c-Src inhibition on cell cycle progression, we assayed the effect of different doses of PP2 on progression through the S-phase. MCF-7 cells were treated with PP2 for 24 h in the presence or absence of tamoxifen. Increasing the PP2 dose led to a proportional decrease in S-phase progression, reaching a plateau at 5 μM PP2 (Fig. 3). This dose-dependent effect of PP2 occurred in the presence or absence of tamoxifen. However, addition of tamoxifen enhanced the block in cell cycle progression at all doses of PP2. While cell cycle progression in CSS with 5 μM PP2 was $3.32 \pm 0.28\%$ of control (no PP2) cells, addition of tamoxifen to 5 μM PP2 was only $1.88 \pm 0.35\%$ of control cells. Thus, tamoxifen, upon combination with PP2, led to a significant decrease (~44%) ($P = 0.0058$) in cell cycle progression over CSS and PP2. Therefore, these results suggest that simultaneous inhibition of c-Src and ER block cell cycle progression more effectively.

Estrogen-dependent proliferation is mediated by two key cell cycle regulatory proteins, c-Myc, and cyclin D1 [26]. For this reason we compared changes in expression of these proteins in MCF-7 cells treated with hormonal therapy in the presence or absence of PP2. Inhibiting c-Src decreased the levels of cyclin D1 and c-Myc protein in all treatments (Fig. 4). Quantification of the levels of c-Myc and cyclin D1 indicated that tamoxifen and estrogen-deprivation consistently reduced c-Myc expression while their effect on cyclin D1 expression was more variable, specially for estrogen-deprivation alone (5% CSS). This variability of cyclin D1 expression after estrogen-deprivation may be

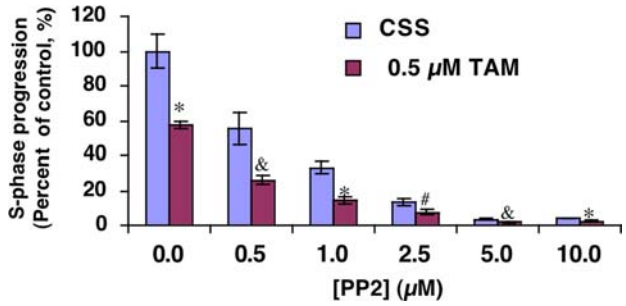

Fig. 3 Effect of different doses of PP2 on cell cycle progression. MCF-7 cells were treated with 5% CSS alone or in the presence of 0.5 μM tamoxifen and the indicated doses of PP2. Cell cycle progression through the S-phase of the cell cycle was evaluated using thymidine incorporation after 1 day of treatment. Results are representative of at least five independent experiments. *$P < 0.005$; & $P < 0.01$; #$P < 0.05$

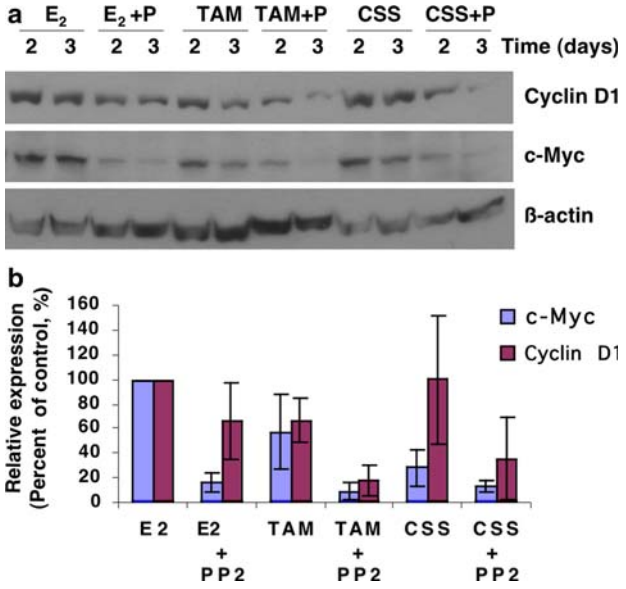

Fig. 4 Expression of cyclin D1 and c-Myc after inhibition of c-Src. MCF-7 cells were treated with maintenance media (FBS), 5% CSS alone (*CSS*) or with 1 μM tamoxifen (*Tam*) or 5 nM 17β-estradiol (E_2) in the presence or absence of 5 μM PP2. Cells were harvested at the indicated days and analyzed for expression of cyclin D1 and c-Myc by Western blot (**a**). β-Actin was used as a loading control. The results are representative of at least five independent experiments. Relative quantification of c-Myc and cyclin D1 expression (**b**). Values represent the average ± SD of c-Myc ($n = 4$) and cyclin D1 ($n = 6$) expression on day 3 compared to estrogen-treated cells (*control*)

the result of the presence of growth factors in CSS inducing cyclin D1 but not c-Myc [27], or due to residual levels of estrogen from the plating media. However, the lowest levels of these ER targets were observed when MCF-7 cells were treated simultaneously with PP2 (5 μM) and ER blockade. Thus, these data suggested that inhibition of c-Src family kinases blocked cell cycle progression by promoting downregulation of c-Myc and cyclin D1.

Effect of tamoxifen and inhibition of c-Src family kinases on cell survival

From our growth curve data in Fig. 1, we observed a decrease in overall cell growth in cells treated simultaneously with tamoxifen and PP2. To determine whether cells were dying from apoptosis, we fixed cells treated with this combination therapy and evaluated the presence of two markers of apoptosis: Bax relocalization to the mitochondria and chromatin condensation [28]. We assessed the presence of these two apoptotic markers in cells treated with tamoxifen and PP2 by using antibodies against Bax and DAPI as a DNA stain. The proapoptotic molecule Bax moves from the cytosol to the mitochondria during early stages of apoptosis giving rise to a punctate staining pattern [28]. Later on, nuclear changes take place leading to chromatin condensation followed by nuclear fragmentation [28]. MCF-7 cells treated with tamoxifen and PP2 showed features characteristic of apoptotic cells as shown by punctate expression of Bax alone (early apoptosis) or accompanied by chromatin condensation (Fig. 5a). In addition, cells treated with tamoxifen and PP2 showed reduced levels of full length PARP (116 kDa) and increased levels of the cleaved form of PARP (85 kDa), another marker of apoptotic cell death (Fig. 5b). Thus, our data show that cells treated with tamoxifen and PP2 die from apoptosis.

To establish the pathways leading to apoptosis in MCF-7 cells treated with the combination of tamoxifen and PP2, we evaluated changes in expression of Bcl-2 family members that regulate apoptosis. Bcl-2 is a known target of ER [29–34]. To our surprise, in contrast to what we observed with the ER targets c-Myc and cyclin D1, addition of PP2 to tamoxifen treated cells led to upregulation of Bcl-2, whereas levels of Bcl-x_L, Bcl-x_S, Bak, or Bax were unchanged (Fig. 6a). As Mcl-1 is a downstream target of c-Src, we determined the changes in Mcl-1 expression upon inhibition of c-Src kinase [35]. Although, in some instances, we observed a decrease in the full-length form of Mcl-1 (40 kDa), an increase in a smaller form of Mcl-1 (28 kDa) occurred more consistently (Fig. 6b). It is known that smaller forms of Mcl-1 can loose their ability to promote survival and may, instead, play a proapoptotic role [36]. Thus, these findings suggest that changes in the isoform ratio of Mcl-1 might favor apoptotic cell death in cells treated with PP2 and hormonal therapy.

Recently, a role of Bad in mediating estrogen-dependent survival in serum-deprived cells has been

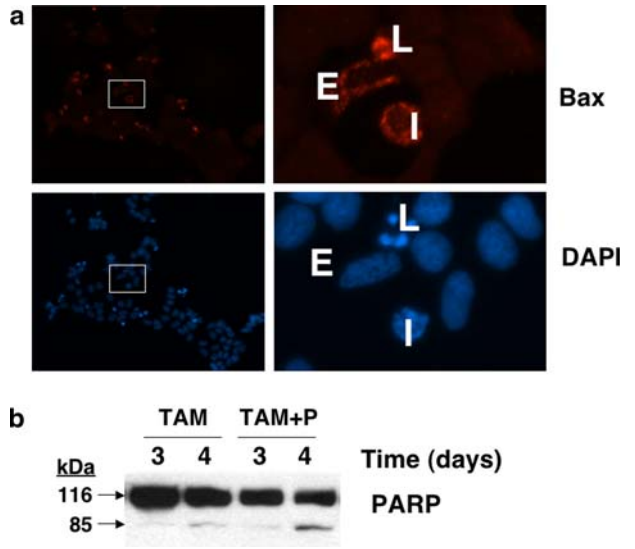

Fig. 5 Apoptotic features in cells treated with tamoxifen and PP2. MCF-7 cells were treated with 5% CSS and 1 μM tamoxifen in the presence of 5 μM PP2. **a** After 4 days of treatment, cells were fixed and stained for Bax (*top*) and DNA (*bottom*) (see Materials and methods). *Right panel* enlargement of area within box on respective left panel *E* early apoptotic cell with punctate expression of Bax and no chromatin condensation, *I* intermediate stage of apoptosis with cell shrinking and chromatin condensation, *L* late apoptotic cell with fragmented nuclei. Results are representative of four independent experiments. **b** Detection of full length (116 kDa) and cleaved PARP (85 kDa) by Western blot after treatment with tamoxifen in the presence or absence of PP2

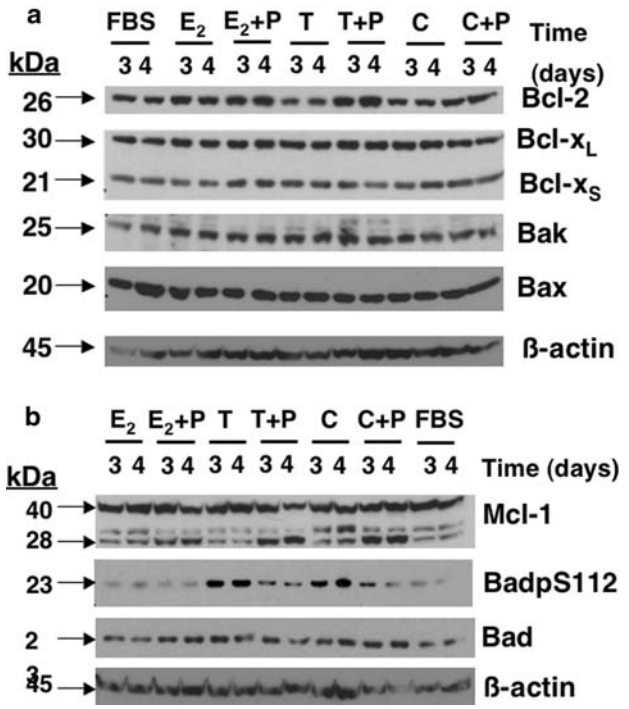

Fig. 6 Changes in expression of Bcl-2 family members after c-Src kinase blockade. MCF-7 cells were treated with maintenance media (*FBS*), 5% CSS alone (*C*) or with 1 μM tamoxifen (*T*) or 5 nM 17β-estradiol (E_2) in the presence or absence of 5 μM PP2. Cells were harvested on the days indicated and analyzed for the expression of Bcl-2, Bcl-x (Bcl-x_L and Bcl-x_S), Bak, and Bax (**a**) or Mcl-1, Bad and BadpS112 (**b**) by Western blot. β-actin was used as a loading control. The molecular weights of the indicated protein bands are shown on the left hand side of each autoradiogram. The results are representative of at least three independent experiments

proposed [37]. Specifically, in serum-deprived MCF-7 cells short treatment with estrogen can promote phosphorylation of Bad at serine 112 (BadpS112), leading to its inactivation as a proapototic molecule [37]. Therefore, we analyzed expression of Bad and its phosphorylated form. To our surprise, in serum containing media, treatment with tamoxifen or estrogen-deprivation led to higher levels of BadpS112 compared to cells grown in their regular growth medium (FBS) or in CSS supplemented with estrogen (Fig. 6b). Nevertheless, inhibition of c-Src kinase decreased the levels of BadpS112 in MCF-7 cells treated with tamoxifen or estrogen-deprivation but not in estrogen-treated cells. Total levels of Bad were unaffected by inhibition of c-Src (Fig. 6b). Thus, the decreases in Bad phosphorylation may promote apoptotic death in cells treated with PP2 and hormonal therapy. The lack of changes in BadpS112 after c-Src blockade in estrogen-treated cells uncovered a crucial role for c-Src on survival of ER-positive breast cancer cells with impaired ER function. Therefore, our results suggest that combining c-Src and ER blockade may lead to death of ER-positive breast cancer cells.

Discussion

Novel targeted therapies against tyrosine kinases are being developed for different types of cancer including breast cancer. For example, several compounds against the non-receptor tyrosine kinase c-Src are currently in clinical trials [11, 12, 38]. Although use of these new drugs alone may already be of benefit to patients, it is possible that combining these novel drugs with existing therapies may provide a greater clinical benefit in prolonging the life of patients and preserving their quality of life. To our knowledge, the interaction between hormonal therapy and c-Src inhibition has not been previously described. In this study, we show that blocking c-Src family tyrosine kinases cooperates with inhibition of ER function in decreasing cell cycle progression and survival of ER-positive MCF-7 cells. These results suggest that therapeutic strategies to target both ER and c-Src pathways simultaneously may lead to better clinical responses than either therapy

alone. In particular, we provide evidence that combination therapy effectively inhibits cell growth by inducing cell cycle arrest and cell loss via apoptosis.

As c-Src can mediate estrogen-dependent proliferation [15, 17], blocking c-Src function with the compound PP2 should prevent estrogen-dependent cell cycle progression. Indeed, we observed that MCF-7 cells supplemented with estrogen could not grow in the absence of c-Src kinase function. Similarly, MCF-7 cells could not grow in the presence of tamoxifen or without estrogen supplementation (estrogen-deprivation). However, when hormonal treatments were combined with inhibition of c-Src family tyrosine kinase, besides blocking of cell proliferation, a decrease in overall cell survival was observed. These results uncovered a crucial role for c-Src in the survival of ER-positive cells treated with hormonal therapy.

Our observations that inhibition of c-Src family tyrosine kinases enhanced the effect of tamoxifen on cell cycle progression led us to compare levels of the ER targets c-Myc and cyclin D1 in tamoxifen treated cells with or without PP2. Both ER targets were reduced by PP2 in ER-positive MCF-7 cells. Previously, c-Src has been shown to be required for estrogen-dependent cyclin D1 expression [15]. In addition, c-Src can regulate c-Myc expression [39]. Thus, it is not surprising that blocking c-Src activity leads to downregulation of both cyclin D1 and c-Myc. Therefore, the ability to downregulate both of these key cell cycle regulatory proteins can explain the efficacy of c-Src inhibition in blocking cell cycle progression in ER-positive breast cancer cells.

Although blocking cell proliferation is a valid therapeutic strategy to control tumor growth, tumor cells can acquire drug resistance leading to tumor growth, despite the presence of the drug. Thus, a better therapeutic strategy is to induce death of tumor cells. Our data indicate that simultaneous blockage of ER and c-Src function leads to cell loss by apoptosis. In this case, induction of cell loss was not related to downregulation of Bcl-2, since we observed that c-Src blockade, instead, increased Bcl-2 expression. It is not clear why c-Src blockade enhanced Bcl-2 expression. One possible explanation is that upregulation of Bcl-2 is an indirect effect of c-Src blockade due to c-myc downregulation, as c-Myc is known to repress Bcl-2 expression [40]. However, Bcl-2 activity is regulated by post-transcriptional modifications [41]. Further experiments will be needed to determine whether Bcl-2 is still active as survival factor upon c-Src blockade. If so, additional therapy to block Bcl-2 function with BH3 mimetics such as ABT-737 [42] may counteract this compensatory survival pathways induced upon c-Src blockade and enhance cell death further.

The induction of apoptosis in cells treated with tamoxifen and the c-Src inhibitor may, partially, depend on changes in the survival factor Mcl-1. In melanoma cells, inhibition of c-Src family tyrosine kinases downregulates expression of the full-length form of Mcl-1 [35]. Although no changes on the expression of small isoforms of Mcl-1 were described in the indicated study, it is possible that melanoma cells do not express these other Mcl-1 isoforms. Several small isoforms have been reported, and their expression differs according to cell type and stimuli [36, 43–47]. The origin of these several small isoforms is also diverse (differential exon-usage or proteolytic digest). In general, these small isoforms of Mcl-1 have either lost their antiapoptotic properties or have become proapoptotic [36, 43–47]. Therefore, a change in the ratio of proapoptic/antiapototic Mcl-1 could play a role in promoting apoptosis upon c-Src blockade.

An additional mechanism that may promote apoptosis in MCF-7 cells treated with hormonal therapy and PP2 is a decrease in the inactive form of Bad when ER and c-Src are simultaneously blocked compared to ER blockade alone. Decreases in BadpS112 (non-proapoptotic), despite similar levels of Bad expression, will again increase the ratio of proapoptic/antiapototic molecules, thereby promoting apoptosis. We observed upregulation of BadpS112 when cells were treated with estrogen-deprivation or tamoxifen. It is possible that upregulation of BadpS112 is mediated by serum factors or adhesion-dependent signals via c-Src and promote survival ("compensatory survival") of ER-positive cells during estrogen-deprivation or tamoxifen therapy [48]. The survival of breast cancer cells after hormonal therapy can give rise to a dormant population of cells that with time can adapt to the specific hormonal therapy and resume growth. Our findings that c-Src blockade decrease the levels of BadpS112 only when ER function is also blocked, indicate that c-Src function is crucial for the survival of ER-positive breast cancer cells treated with hormonal therapy. In support of a specific role of c-Src in specific cell contexts, recent studies using a temperature-sensitive c-Src or v-Src transformed cell line have shown that c-Src function becomes "crucial" for survival of these transformed cells only after serum withdrawal [49]. In parallel to these observations, we propose that endogenous c-Src function becomes "crucial" for ER-positive breast cancer cells only when ER function is blocked. Therefore, our data suggest that targeting both ER and c-Src function may promote death of ER-positive breast cancer.

In the clinic, use of c-Src inhibitors in combination with hormonal therapies may prolong time to disease progression by inducing death of tumor cells and

preventing acquisition of hormonal resistance. The potential role of c-Src in driving acquisition of hormonal resistance was suggested by two recent studies showing overexpression of the active form of c-Src when MCF-7 cells acquire resistance to tamoxifen [4, 5]. Inhibition of c-Src in tamoxifen-resistant cells impairs cell proliferation [5] and cell motility [4]. Since overexpression of active c-Src is frequent in metastatic ER-positive breast cancer [5], use of c-Src inhibitors in combination with hormonal therapy may benefit patients with advanced breast cancer and, if accompanied by low toxicity, may also be useful in the adjuvant or preventive setting. Moreover, as the agonist effect of tamoxifen in uterine tissue may also require c-Src, combining targeted therapy against c-Src and tamoxifen may prevent the increased risk of uterine cancer associated with tamoxifen [13, 14]. Alternatively, due to the role of c-Src in bone remodeling [50], the use of c-Src with estrogen-deprivation (aromatase inhibitors) may reduce the risk of osteoporosis. In conclusion, all these studies suggest that the use of hormonal therapy in combination with inhibition of c-Src is a valid therapeutic strategy for ER-positive breast cancer.

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