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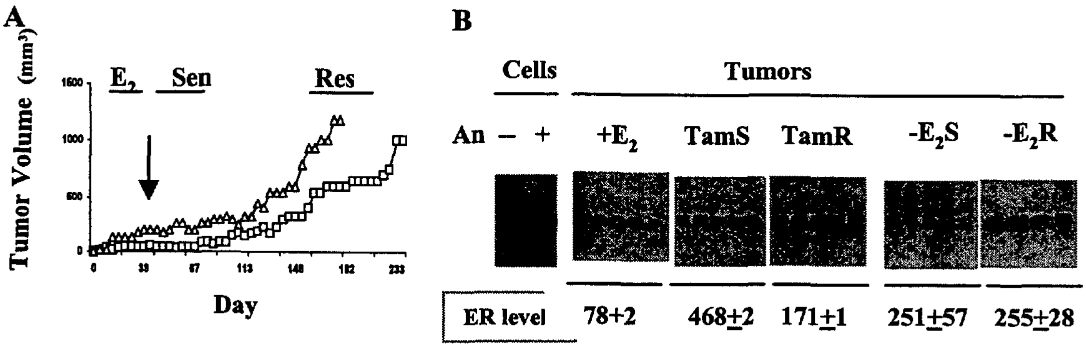
(54) Title: P38 MAPK PATHWAY PREDICTS ENDOCRINE-RESISTANT GROWTH OF HUMAN BREAST CANCER AND PROVIDES A NOVEL DIAGNOSTIC AND TREATMENT TARGET



(57) Abstract: Acquired and *de novo* endocrine resistance are major clinical problems in the management of breast cancer patients. Though the antiestrogen tamoxifen prolongs disease-free and overall survival in the adjuvant setting, and induces remissions in over half of the patients with estrogen receptor positive metastatic disease, all patients eventually acquire tamoxifen resistance. Furthermore, many of the resistant tumors actually appear to be stimulated by tamoxifen just as they are by estrogens. The present invention provides methods of predicting endocrine resistance comprising detecting the biological activity and/or expression of p38 MAPK and/or AIB1. The invention further provides methods of reducing, reversing, or preventing endocrine resistance comprising contacting a breast or prostate tumor with a p38 MAPK pathway inhibitor.

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P38 MAPK PATHWAY PREDICTS ENDOCRINE-RESISTANT GROWTH OF HUMAN BREAST CANCER AND PROVIDES A NOVEL DIAGNOSTIC AND TREATMENT TARGET

BACKGROUND OF INVENTION

This application claims the benefit of U.S. Provisional Application Serial No. 60/299,824 filed on June 21, 2001.

This work was supported by a grant from the National Institutes of Health/National Cancer Institute grant number P50 CA58183. Accordingly, the government has certain rights in the invention.

Breast cancer development and progression are influenced by steroid hormones, particularly estrogen, via their interaction with specific target cell receptors. Tamoxifen is a non-steroidal antiestrogen which is now the most frequently used drug in breast cancer treatment. Tamoxifen is thought to inhibit breast cancer growth by competitively blocking estrogen receptor (ER), thereby inhibiting estrogen-induced growth. ER, a ligand-dependent transcription factor, meditates most biological effects of estrogens on cell and tumor growth [Katzenellenbogen et al., 2000, *Recent Prog Norm Res* 55:163-195]. In the adjuvant setting after primary surgery for breast cancer, tamoxifen has been shown to prolong disease-free and overall survival, and it has also been shown to induce remissions in more than half of patients with metastatic disease who have ER-positive tumors [Early Breast Cancer Trials Collaborative Group, 1992, *Lancet* 339:1-14, 71-85; Saez et al., 1989, *Current Clinical Oncology*, Alan R Liss Inc., New York, pp.163-172]. Although tamoxifen is initially effective in many patients, 50% of the patients fail to respond to the drug despite the presence of ER. Furthermore, even patients who initially respond eventually acquire tamoxifen resistance, leading to tumor progression and death. The mechanisms for either intrinsic or acquired tamoxifen resistance are unknown, but they are probably multifactorial.

In both animal models and clinical specimens, lower tamoxifen uptake and somewhat altered tamoxifen metabolism in resistant tumors have been observed, but neither appears to explain tamoxifen stimulation of the resistant tumors. Nor do estrogen receptor losses or mutations appear to explain this phenomenon, although altered expression of transcriptional variant forms of the receptor may well contribute. Pure steroidal antiestrogens such as ICI 182,780 are capable of reversing tamoxifen-stimulated as well as estrogen-stimulated growth of these resistant tumors, and are now in clinical trials for this purpose [Osborne et al., 1994, *Breast Cancer Res Treat.* 32(1):49-55]. Endocrine treatment of breast cancer is the major form of systemic adjuvant therapy and therapy for metastatic disease. Unfortunately, however, not all tumors will respond, and furthermore, initially responding tumors eventually become resistant to the endocrine treatment, leading to tumor progression and death.

The present inventors are interested in the mechanisms by which tumors develop resistance to tamoxifen, with the ultimate goal of developing new strategies for preventing or reversing the emergence of resistant cells. There are several possible mechanism by which tamoxifen resistance could develop in breast cancer cells. Clues to these mechanisms can be gleaned from an understanding of the myriad effects that tamoxifen has at the cellular level, as outlined in Figure 1. Tamoxifen binds to the ER and competitively blocks estrogen-induced transcription of specific genes encoding proteins involved with regulation of cell proliferation. Some of these proteins are in fact polypeptide growth factors, such as transforming growth factor α, insulin-like growth factor II, and members of the fibroblast growth factor family, which by autocrine and paracrine mechanisms may enhance tumor growth. Down-regulation of the gene expression of these growth factors by tamoxifen may result in suppression of tumor growth. Oddly enough, breast cancer cells, as well as other tumor cells, may also synthesize and secrete growth inhibitors, such as transforming growth factor-β. Expression of TGFβ is reduced by estrogen, but enhanced by tamoxifen treatment. Thus, increased expression of growth inhibitors by tamoxifen may also contribute to tumor growth suppression. Clearly, alterations in the expression of these growth factors or growth inhibitors, or their specific cell membrane receptors, could provide the tumor cell with sufficient growth stimulation to overcome the tamoxifen block, resulting in tamoxifen resistance. Cross-talk between polypeptide growth factor pathways and ER-mediated events could also theoretically result in tamoxifen resistance. It has been shown, for example, that increasing the level of cellular cyclic AMP pharmacologically alters the cellular response to tamoxifen, converting it from an antiestrogen to a weak estrogen agonist [Fujimoto et al., 1994, *Mol Endocrinol.* 8(3):296-304]. The mechanism for this phenomenon is not yet understood, but it could be related to changes in the phosphorylation state of the ER itself and/or its coregulatory protein.

Other potential mechanisms for the development of tamoxifen resistance include the loss of or mutations in the ER, or altered expression of the accessory proteins that could modify the transcriptional signal generated by the ligands binding to estrogen receptor. Also, since certain metabolites of tamoxifen are known to be less antiestrogenic, or even to be full estrogen agonists, changed systemic metabolism of tamoxifen or altered uptake or metabolism of tamoxifen in the tumor itself could also result in tamoxifen resistance. Finally, high levels of the so-called antiestrogen binding sites, cytoplasmic binding sites whose function is not yet well understood, could theoretically serve as a sump, soaking up tamoxifen molecules and preventing their binding to ER. Studies of several of these possibilities have been initiated in laboratory models.

Clinical studies with tamoxifen provide several clues for mechanisms by which acquired resistance may develop. Patients whose tumors initially lack ER have a very low response rate to the drug, and thus, selection of an ER-negative clone of tumor cells could result in an estrogen-independent tumor refractory to tamoxifen. Some patients with tamoxifen resistance do develop resistance to all forms of endocrine therapy via selection of an ER-negative tumor cell clone. However, it was recently reported that a series of patients with acquired tamoxifen resistance in whom tumor estrogen and progesterone receptors were measured by both ligand binding and immunohistochemical assays (to circumvent the problem of receptor occupancy by the drug) [Encarnacion et al., 1993, *Breast Cancer Res. Treat.* 26(3):237-246]. More than 60% of tumors continued to express ER and/or PgR even while progressing in the face of tamoxifen. These data indicate that while ER negativity may account for some cases of resistance, mechanisms of resistance other than receptor loss must be common.

If patients' tumors remain ER-positive after development of tamoxifen resistance, one might expect that some of these tumors have retained estrogen sensitivity and will respond to other endocrine treatments. In fact, clinical experience demonstrates that patients who have initially responded to tamoxifen but who later develop tumor progression, frequently respond to second or third-line endocrine therapies. Thus, acquired tamoxifen resistance in these patients does not necessarily indicate global hormonal unresponsiveness, but rather selective resistance to tamoxifen itself. Although it has not been studied systematically, anecdotal experience suggests that some patients with tamoxifen resistance will respond to a rechallenge with the drug after an interval in which they receive other treatments. Furthermore, clinical reports suggest that patients who receive tamoxifen *adjuvant* therapy and then later recur, not infrequently will respond to a rechallenge with the drug. This suggests that tamoxifen resistance, in some cases, may not be a permanent phenotype, but rather may be reversible when administration of the drug is stopped. Patients may also respond to an increase in the tamoxifen dose after developing progression with a lower dose schedule. Finally, similar to reports of patients treated with high dose estrogen therapy, some patients who have responded to tamoxifen will have a withdrawal response when the drug is stopped at the time of tumor progression. The prolonged half-life of tamoxifen makes it difficult for clinicians to withhold alternative therapy while waiting for a withdrawal response to the drug. Nevertheless, these data strongly suggest that in some patients with acquired resistance, tamoxifen may actually be stimulating tumor growth.

Two previously published clinical trials also suggest that tamoxifen-stimulated tumor growth may be a cause of tamoxifen resistance in some patients [Pritchard et al., 1980, *Cancer Treat Rep.* 64(6-7):787-96][Hoogstraten et al., 1984, *Cancer* 54(10):2248-2256]. In these studies, premenopausal women with advanced breast cancer were treated with second-line ovarian ablation after they first responded and then progressed on tamoxifen. In one of these studies, the secondary response to ovarian ablation was common in patients who had previously responded to tamoxifen, suggesting that tamoxifen treatment served as an *in vivo* tumor estrogen sensitivity assay. However, in the other study, opposite results were obtained and no patients responded to second-line ovarian ablation. In this latter study tamoxifen therapy was continued after the surgery, while in the first study tamoxifen treatment was stopped. Secondary response to ovarian ablation would not be expected in the latter study if tamoxifen itself was behaving as an estrogen agonist and stimulating tumor growth. Tamoxifen-stimulated tumor growth as a mechanism for acquired resistance is further supported by data from the present inventor's laboratory as well as others using experimental models. A major focus of the present inventor's group is to better understand mechanisms by which tamoxifen-stimulated tumor growth occurs.

SUMMARY OF THE INVENTION

Endocrine treatment of breast cancer is the major form of systemic adjuvant therapy and therapy for metastatic disease. Unfortunately not all tumors respond, and furthermore, initially responding tumors eventually become resistant to endocrine therapies [Osborne, 1998, *N Engl J Med* 339:1609-1618]. This may lead to tumor progression and death. Identifying the factors and pathways responsible for the development of this resistance and defining ways to overcome this resistance are, therefore, important diagnostic and therapeutic goals in breast cancer research.

The present invention provides a method of predicting resistance to endocrine treatment comprising providing a biological sample, *e.g.* a breast tumor biopsy, and detecting the activity and/or expression of p38 MAPK and/or AIB1.

The present invention further provides a method for treating breast cancer comprising contacting a breast tumor with a p38 MAPK inhibitor. The p38 MAPK inhibitor may be administered to a subject and may be combined with an endocrine therapy. Administration of the p38 MAPK inhibitor may ameliorate, reverse or prevent endocrine resistance. Thus, the present invention provides a method of ameliorating or reversing *de novo* endocrine resistance and a method of ameliorating, reversing or preventing acquired endocrine-resistance in an endocrine-resistant tumor.

The present invention further provides a method of treating a breast cancer comprising contacting a breast tumor with an inhibitor of the p38 MAPK pathway including inhibitors that act upstream or downstream of p38 MAPK.

Use of the therapeutic methods of the invention should improve survival of breast cancer patients.

The invention further relates to drug screening assays designed to identify compounds that modulate p38 MAPK activity and the use of such compounds in the treatment of disorders such as cancer. The invention also relates to methods for diagnosis and prognosis of disorders such as cancer that rely on detection of activation or overexpression of p38 MAPK. In one preferred embodiment, the invention relates to methods for diagnosis an prognosis of breast cancer and/or prostrate cancer.

The invention is supported by the discovery that p38 MAPK is up-regulated when breast tumors develop resistance to the endocrine treatments such as estrogen withdrawal or tamoxifen. Up-regulation of p38 MAPK has been observed in an athymic mouse xenograft model of endocrine resistance (*e.g.* Example 2, below) and in a survey of over 150 biopsies of tamoxifen resistant breast tumors. This invention is further supported by the discovery that p38 MAPK is upregulated in breast tumors that display *de novo* tamoxifen resistance.

Thus, patient tissue may be assayed for levels of p38 MAPK activity or expression, wherein increased levels of p38 MAPK activity or expression signify an increased likelihood of resistance to endocrine treatments. In addition, the assays of the invention may be used to monitor the progression of the disease within a patient.

The invention further relates to assays designed to screen for compounds that modulate the biological activity of p38 MAPK (*e.g.* catalytic activity) or genes and gene products upstream or downstream to p38 MAPK. The invention also relates to assays designed to screen for compounds that modulate p38 MAPK gene expression. For example, cell-based assays may be used to screen for compounds that modulate p38 MAPK transcription such as compounds that modulate expression, production or activity of transcription factors involved in p38 MAPK gene expression; antisense and ribozyme polynucleotides that modulate translation of p38 MAPK mRNA and polynucleotides that form triple helical structures with the p38 MAPK regulatory region and inhibit transcription of the p38 MAPK gene.

Identified compounds may be used in the treatment of disorders such as cancer including breast and ovarian cancer where activity or transcription of p38 MAPK contributes to tumor development or growth or resistance to therapy.

The invention also provides p38 MAPK inhibitors and p38 MAPK pathway inhibitors that may be used to ameliorate or prevent *de novo* or acquired estrogen resistance. In some embodiments of the invention, the amount of a p38 MAPK inhibitor administered to a subject is from about 1 mg/kg to about 100 mg/kg. In some embodiments, the amount is from about 5 mg/kg to about 60 mg/kg. In some embodiments administration of a p38 MAPK inhibitor is coextensive with administration of an endocrine agent. In other embodiments of the invention, p38 MAPK inhibitor administration begins before or after endocrine administration begins.

Inhibitors of the invention may be small organic molecules, antibodies, or nucleic acids that inhibit the biological activity of a member of the p38 MAPK pathway. Inhibition may be accomplished by any means including reducing catalytic activity (*e.g.* small molecule inhibitors), reducing protein availability (*e.g.* antibodies), reducing gene product formation (*e.g.* antisense nucleic acids), and increasing gene product turnover (*e.g.* dephosphorylation, ubiquitination).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Schematic diagram of the mechanism of tamoxifen action.

Figure 2: Panel A shows the development of acquired resistance in xenografted tumors treated with E2 withdrawal (Δ) or with Tam (□). Panel B shows p38 MAPK activity as measured by Western blots using an antibody that recognizes the active (phosphorylated) form of p38. ER levels in tumors (fmol/mg protein) are shown.

Figure 3: *In vitro* phosphorylation of ERα by p38 MAPK. Panel A shows increasing doses of purified (recombinant) p38 MAP kinase phosphorylate baculovirus-expressed ERα. "SB" – p38-specific inhibitor SB 203580. Panel B shows phosphorylation of GST-fusion constructs of the AF1 and Hinge domains of the ER by recombinant p38 MAPK. Panel C shows phosphorylation of ERβ.

Figure 4: *In vitro* phosphorylation of Xenopus oocyte-expressed AIB1 by p38 MAPK.

DETAILED DESCRIPTION OF THE INVENTION

According to the instant invention, the term "endocrine therapy" means any therapy designed to interrupt the signal generated by estrogen binding to estrogen receptor. Nonlimiting examples include treatments which lower the estrogen level and treatments designed to block the estrogen receptor such as administration of the drug tamoxifen.

According to the instant invention, the term "estrogen withdrawal" means any therapy designed to reduce the level of estrogen in a subject. Estrogen withdrawal may be accomplished by ovarian ablation or surgical removal. Estrogen withdrawal also may be accomplished by administering to the subject a molecule that inhibits estrogen formation or enhances estrogen degradation. Estrogen withdrawal also may be accomplished by administering to the subject a molecule that converts estrogen to an inactive form.

According to the instant invention, the term "p38 MAPK inhibitor" means any material that blocks signaling through the p38 MAP kinase pathway. In some embodiments of the invention, p38 MAPK inhibitors function by reducing the amount of p38 MAPK, inhibiting or blocking p38 MAPK activation, or inhibiting other molecules in the signaling pathway.

Non-limiting examples of p38 MAPK inhibitors include antisense p38 MAPK nucleic acids and fragments thereof. Further non-limiting examples of p38 MAPK inhibitors include antibodies that bind p38 MAPK and fragments thereof. Still further non-limiting examples of p38 MAPK inhibitors include EO-1428, PD169316, SB202190, SB203580, SB239063 (Legos et al., 2001, Brain Res. 892:70-77; Barone et al., 2001, Med Res Rev. 21(2):129-145), SB281832, VX-702, VX-745, ZM336372, RPR 200765A (Mclay LM et al., 2001, Bioorg Med Chem. 9(2):537-554), and N-(3-tert-butyl-1-methyl-5-pyrazolyl)-N'-(4-(4-pyridinylmethyl)phenyl)urea (Dumas J, 2002, Bioorg Med Chem Lett 12(12):1559-62).

According to the instant invention, the term "p38 MAPK activity" means the ability of p38 MAPK to phosphorylate, *inter alia*, amplified in breast cancer 1 (AIB1), activating transcription factor 2 (ATF-2), estrogen receptor α (ERα), estrogen receptor β (ERβ), mitogen activated protein kinase activated protein kinase 2 (MAPKAP-K2), MAPKAP-K3, p38-related/activated protein kinase (PRAK), Menkes copper transporting P-type ATPase (MNK), mitogen- and stress-activated protein kinase (MSK), ribosomal S6 kinase B (RSK-B), signal transducer and activator of transcription 1 (STAT1), Max/Myc complex, Ets-like transcription factor-1 (Elk1), C/EBP homologous protein (CHOP), myocyte enhancer factor 2 (MEF2), and fragments thereof as measured by any phosphorylation assay known in the art. Nonlimiting examples of phosphorylation assays include activity assays (*e.g.* in-gel kinase assays) and immunologic assays (*e.g.* use of an antibody that specifically binds to the phosphorylated protein in immunohistochemistry, immunofluorescence, Western blotting, and ELISA).

According to the instant invention, the term "p38 MAPK expression" means the formation of a p38 MAPK gene product as measured by any method known in the art including nucleic acid hybridization method, *e.g.* Northern blotting, *in situ* hybridization; nuclear run-on assays; polymerase chain reaction amplification; reporter gene expression, *i.e.* where the reporter gene is operatively linked to p38 MAPK expression control sequences; gene expression arrays. Formation of a p38 MAPK gene product may also be measured by any antibody-based technique including immunohistochemistry, immunofluorescence, Western blotting, and ELISA.

According to the instant invention, the term "providing a biological sample" means supplying cells, fluids, tissues and/or organs by any means such that said cells, fluids, tissues, and/or organs are suitable for p38 MAPK pathway activity and/or expression analysis. In some embodiments of the invention, the biological sample is biopsied, resected, drawn or otherwise harvested from a subject. In other embodiments of the invention, the biological sample is presented for analysis within its native *in vivo* context. A nonlimiting example is novel magnetic resonance imaging (Jacobs et al., 2001, J Nucl Med 42(3):467-475; Wunderbaldinger et al., 2000, Eur J Radiol 34(3):156-165) techniques wherein the biological sample may be identified and subjected to p38 MAPK pathway activity and/or expression analysis while remaining in a living subject throughout. Expression of p38 MAPK pathway genes may be detected using a nucleic acid construct comprising a p38 MAPK pathway gene expression control sequence operatively linked to a reporter gene. This nucleic acid may be delivered to cells *in vitro* or *in vivo* using particle bombardment [U.S. Pat. No. 5,836,905 to Lemelson et al.] or any other delivery technique known in the art.

According to the instant invention, the term "resistance", *i.e.* endocrine resistance, estrogen withdrawal resistance or tamoxifen resistance means that a tumor does not regress (get smaller) or have prolonged stable disease (no change in size) when a new treatment is initiated. In other words the tumor maintains its exact size for a minimum of 6 months, a condition called stable disease, or it regresses by more than 50% in volume, which is a response. Tumors that do not qualify for either one of these are *de novo* resistant to the treatment. For acquired resistance, tumors initially regress and get smaller, but then as they become resistant the begin to regrow. The standard definition is that the tumor volume increases by 25%. This indicates that the treatment is no longer working and another therapy must be substituted. Thus, resistance is a clinical term used to indicate that the that the treatment is not working because the tumor is progressing. *De novo* resistance can usually be detected within the first 4 to 8 weeks of treatment. If the tumor is going to respond to the treatment then it will usually stop growing or actually regress by this time. For acquired resistance the tumor remains stable or regresses in size for a minimum of size months in order for it to be called a response. The indicator of the development of acquired resistance is when the tumor progresses after this period of response and begins to regrow.

Current endocrine therapies of breast cancer are based mainly on targeting the estrogen receptor (ER) signaling pathway by either reducing levels of estrogen or by antagonizing ER function with antiestrogens such as Tam. There are numerous prognostic markers in breast cancer, but there are few biological markers which predict response to treatment. Furthermore, though ER status itself is a vital factor and therefore a predictor of response to hormone therapy, it nevertheless fails to predict the *de novo* and acquired resistance that occurs in a significant portion of ER-positive patients. It is known that different growth factor pathways and their downstream MAPKs can modulate endocrine response. However, the underlying molecular events that contribute to endocrine resistance of breast cancer are yet unknown. Identifying the factors and pathways responsible for the development of this resistance and defining ways to overcome this resistance are, therefore, important diagnostic and therapeutic goals in breast cancer management.

Therefore, what is unique about the present inventor's discovery is that the p38 MAPK pathway, which has been implicated in inflammation and cellular stress, predicts response to endocrine treatment in breast cancer; specifically, its activation and/or increased expression is associated with the development of resistance to common treatments in breast cancer, namely antiestrogens and estrogen withdrawal. The clinical implication is that endocrine-resistant tumor growth might be prevented with inhibitors of p38 MAPK (or other proteins in this pathway) used simultaneously with the endocrine treatment or added later at the first sign of tumor resistance. Measurements of expression and/or activity of p38 MAPKs (or other proteins on this pathway) might also serve as diagnostic markers to select patients for endocrine therapy.

While the literature has suggested that ERK 1,2 MAPK, as well as other MAPK pathways, are able to phosphorylate and modulate activity of the ER [Kato et al., 1995, Science 270(5241):1491-1494], the association of acquired breast cancer endocrine resistance with the up-regulation of the p38 MAPK pathway is disclosed for the first time by the instant invention.

Various aspects of the invention may be better understood in view of the following detailed descriptions, example, and supporting references.

EXAMPLES

EXAMPLE 1

The present inventors have developed an *in vivo* experimental model of tamoxifen resistance and resistance to estrogen deprivation in which ER-positive MCF-7 human breast cancer cells are inoculated subcutaneously into athymic nude mice [Osborne et al., 1987, *Eur J Cancer Clin Oncol.* 23(8):1189-1196; Osborne et al., 1991, *J Natl Cancer Inst.* 83(20):1477-1482]. Estrogen withdrawal (-E2) or tamoxifen treatment of these mice suppresses tumor growth for several months, but then tumor growth resumes despite continued treatment with the drug [Osborne et al., 1995, *J Natl Cancer Inst* 87:746-750]. This sequence of events is similar to that which occurs in patients. Transplantation of fragments from these tamoxifen-resistant tumors demonstrates that their growth has not become estrogen-independent, but in fact is now stimulated by tamoxifen as well as by estrogen. These data, which have also been reported by others [Katzenellenbogen et al., 2000, *Recent Prog Norm Res* 55:163-195], suggest that one form of acquired tamoxifen resistance may be due to the acquired ability of the tumor cells to be stimulated rather than inhibited by tamoxifen. Since certain metabolites of tamoxifen have estrogenic properties, pharmacologic explanations for this tamoxifen-stimulated growth were investigated first.

Conversion of breast tumors to a (tamoxifen-resistant) Tam^R phenotype, and to lesser extent to (estrogen-deprived-resistant) $-E2^R$, has been shown to be associated with oxidative stress and marked depletion of glutathione levels [Schiff et al., 2000, *J Natl Cancer Inst* 92:1926-1934]. These cellular events may induce stress-induced signaling pathways which could be important in mediating cell proliferation and resuming breast tumor growth. And indeed, in a xenograft model $-E2^R$ and Tam^R resistance have recently been shown to be associated with increased levels of the phosphorylated active form of p38 MAPK, a stress-induced kinase. Importantly also, these resistant tumors have been found to possess high levels of ER and, in addition, that the $-E2^R$ tumors are hypersensitive to low levels of estrogen. Also, it has been shown that $-E2^R$ and Tam^R resistant tumors can still be inhibited by the pure steroidal antiestrogen ICI 182,780 which induces ER degradation and depletion from the cell [Osborne et al., 1995, *J Natl Cancer Inst* 87:746-750]. These findings argue that the resistant-growth of these tumors is mediated through the ER, but that the ER activity has been modulated. ER phosphorylation, which leads to enhancement in receptor activity [Wolgel, 1996, *Biochem J* 319:657-667], is one possible mechanism. Based on the above results, increased activity of p38 MAPK may be an important determinant in the development of endocrine resistance of breast tumors, presumably through direct phosphorylation of ERα, ERβ, and AIB1. Hence, p38 MAPK may serve as a therapeutic target to circumvent endocrine resistance of breast cancer. This is further supported by a recent report which suggested the involvement of p38 MAPK in mediating MEKK1-induced activation of the human ERα and in stimulating the agonistic activity of Tam in endometrial and ovarian cancer cells [Lee et al., 2000, *Mol Endocrinal* 14:1882-1896]. Importantly also, the inventor's data suggests that p38 MAPK can phosphorylate ERα and ERβ *in vitro* and that the AF-1 and hinge domains of the ERα receptor may be involved.

EXAMPLE 2

Methods

ER-positive MCF-7 human breast cancer cells were grown as xenografts in athymic nude mice as described previously [Osborne et al., 1987, *Eur J Cancer Clin Oncol.* 23(8):1189-1196; Osborne et al., 1991, *J Natl Cancer Inst.* 83(20):1477-1482; Osborne et al., 1995, *J Natl Cancer Inst* 87:746-750]. Day 0 was the day of transplantation conducted and the day estrogen treatment began. These mice were treated with estrogen withdrawal (-E2) or tamoxifen (Tam) beginning on day 17. Tumor volume was measure every four days.

Western blots were performed using an antibody that recognizes only the phosphorylated (active) form of p38 MAPK. Tumor fine powder was homogenized on dry ice with lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 10 % Glycerol, 1 mM Pherryl Methylsulfonyl Fluoride, 0.12 mg/mL Antipain-HCl, 0.02 mg/mL Bestatin, 0.04 mg/mL Chrymostatin, 0.12 mg/mL E-64, 0.02 mg/mL Leupeptin, 0.02 mg/mL Pepstatin, 0.12 mg/mL Phosphor-amidon, 0.8 mg/mL pefabloc, 0.02 mg/mL Aprotinin, 1 μM Okadaic acid, 10 μg/mL Microsystin) at ratio 30 mg: 600 μL in pre-chilled glass homogenizer. Powder was then transferred to a prechilled Eppendorf tube. The extract was sonicated four times in 5-second bursts with 5-second intervals between bursts. The sonicated lysate was spun in a benchtop centrifuge at 13,000 rpm, 4° C for 20 min. Clear lysate free of fat was transferred to a new prechilled tube and immediately stored at -80° C.

Aliquots of liquified lysate were subjected to Bradford assay (Bradford, 1976, *Anal. Biochem.* 72:248) to determine protein concentration.

Volumes of lysate sufficient to contain 20 μg each were heated to 95 to 100° C for 5 min in buffer containing 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue. Denatured samples were subjected to standard SDS-PAGE and electrotransferred to a nitrocellulose membrane (0.2 μm, Millipore Inc.).

After transfer, each membrane was washed with 25 mL TBS (1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl) for 5 minutes at room temperature and incubated in 25 mL blocking buffer (1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk). Blocking buffer (150 mL) was prepared by adding 15 mL of 10x TBA to 135 mL water, mixing, adding 7.5 g nonfat dry milk, mixing well, adding 0.15 mL Tween-20 (100%) while mixing, and continuing to mix for an additional hour at room temperature.

Each membrane was then washed three times for 5 minutes each with 15 mL of TBS/T (1X TBS, 0.1% Tween-20).

Each membrane was then incubated in 10 mL primary antibody solution buffer (1X TBS, 0.1% Tween-20 with 5% nonfat dry milk) containing Phospho-p38 MAPK (Thr180/Tyr182) 28B10 Monoclonal Antibody (Cell Signaling Technology, Cat 9216) with gentle agitation overnight at 4°C. Following the overnight primary antibody incubation, each membrane was washed three times for 5 minutes each with 15 ml of TBS/T before incubating with HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature. Each membrane was washed three times for 5 minutes each with 15 ml of TBS/T following the secondary antibody incubation.

Membranes were then incubated with enhanced Chemiluminescent solution (Amershan Pharmacia), drained of excess developing solution, wrapped in plastic wrap and exposed to x-ray film.

Results

Estrogen withdrawal (-E2) or tamoxifen (Tam) treatment suppresses tumor growth for several months ($-E2^S$, Tam^S), but growth eventually resumes as the tumor become resistant to hormonal treatment ($-E2^R$, Tam^R). This is exemplified in Figure 2A, wherein mice with wild type MCF-7 xenografts were treated with E2 withdrawal (Δ) or with Tam (□) and xenografted tumor volume is plotted as a function of time.

Acquired endocrine resistance is a major clinical problem in the management of breast cancer patients, but its mechanism is largely unknown.

In this model of endocrine resistance, p38 MAPK activity and estrogen receptor α (ERα) levels are up-regulated when breast tumors develop resistance to estrogen withdrawal (-E2) or Tamoxifen (Tam). Xenograft tumor extracts from control estrogen-treated (E2), Tam-sensitive (Tam^S), Tam-resistant (Tam^R), -E2-sensitive ($-E2^S$), and -E2-resistant ($-E2^R$) groups were analyzed with an antibody that recognizes the phosphorylated active form of p38 MAPK. Western blots using an antibody that recognizes only the phosphorylated (active) form of p38 MAPK are present in Figure 2B. The left panel of Figure 2B shows controls for phosphorylated p38 MAPK induction, wherein MCF-7 cells were left untreated (-) or treated *in vitro* (+) with 20 µg anisomycin (An) for 30 min., which is known to activate p38 MAPK (+ control). The other five panels each consist of 4 or 5 lanes each loaded with a lance of a separate tumor. Active p38 MAP kinase increases in tumors developing resistance to E2 deprivation (-E2R) or tamoxifen (TamR) compared to the earlier sensitive phases (Figure 2B). ER levels in the tumors (fmol/mg protein) are shown and remain high in the resistant tumors (Figure 2B).

EXAMPLE 3

It is also known that ER is subject to phosphorylation at several sites, and that this modification can cause ligand-independent and/or a synergistic increase in transcriptional activation of ER in the presence of estrogen and antiestrogen. Therefore, increased activity of p38 MAPK may be an important determinant in the development of endocrine resistance of breast tumors, presumably through direct phosphorylation of ERα that enhances the receptor activity in spite of reduced estrogen levels or treatment with antiestrogens such as Tam. Hence, p38 MAPK could serve as a clinical therapeutic target to delay, prevent, or reverse the development of resistance.

The instant invention provides an *in vitro* assay to show that p38 MAPK is capable of phosphorylating ER. Commercially available active recombinant p38 MAPK was used for this Example.

Reactions for Optimization 1 were set up with 1x kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) supplemented with various concentrations of cold ATP and a fixed amount of γ-^{32}P ATP (0.1 μCi, 1 μCi and 5 μCi per 20 μL of total volume, adenosine 5'-triphosphate, ICN Inc., Cat # 35001). These reactions further contained and a constant amount (0.5 μg per 20 μL of total volume) of baculovirus-expressed recombinant human ERα, baculovirus-expressed recombinant human ERβ, GST-fused AF1 of ERα, GST-fused Hinge domain of ERα, GST fused ATF2 protein, or baculovirus-expressed recombinant human AIB1 and active p38 beta (100 ng per 20 μL of total volume; Upstate Biotechology, Cat. # 14-253).

Reactions for Optimization 2 were set up with 1x kinase buffer (as above) supplemented with various concentrations of γ-^{32}P ATP and a fixed amount of cold ATP (10 μM, 100 μM, and 200 μM). These reactions further contained and a constant amount (0.5 μg per 20 μL of total volume) of baculovirus-expressed recombinant human ERα, baculovirus-expressed recombinant human ERβ, or baculovirus-expressed recombinant human AIB1 and active p38 beta (as above).

These reactions under optimized conditions were incubated at 30° C for 30 minutes and then terminated by adding 20 μL 3X SDS sample buffer (187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue) to each and vortexing. Reactions were then spun for 30 seconds in a microcentrifuge.

Samples were heated to 95–100° C for 2–5 minutes and loaded (20 μL per well) on a SDS-PAGE gel (10%).

Following electrophoresis, each gel was fixed with 60% methanol 10% acetic acid for 30 minutes and then stained with Coomassie Blue for 15 minutes. Gels were destained with 10% methanol 10% acetic acid for 2 hours and dried at 70° C for 1 hour in a vacuum dryer. Dried gels were exposed to x-ray film and spot intensities were quantitated.

EXAMPLE 4

The invention further provides data indicating the specific sites on the estrogen receptor that may be phosphorylated by p38. Recombinant p38 MAPK was expressed and purified according to the method of Cuenda A, 1996, EMBO J. 15(16):4156-4164. The AF1 and Hinge domains of the ER were expressed as GST fusion constructs in baculovirus. Fusion proteins were purified and these domains were then assayed according to the *in vitro* phosphorylation assay of Example 3.

Figure 3A shows that increasing doses of purified (recombinant) p38 MAP kinase phosphorylate baculovirus-expressed ERα. This phosphorylation is inhibited in the presence of the p38-specific inhibitor SB 203580 (Figure 3A).

Figure 3B shows that both AF1 (lane 2) and Hinge (lane 3) were phosphorylated by *in vitro* p38 MAP kinase. Lane 1 shows the autophosphorylation of p38 without substrate, which also appears in lane 2 & 3 at lower intensity.

Figure 3C shows the results of incubating reactions containing 0.5 μg recombinant human ERβ, 1 μCi γ-^{32}P ATP, 100 μM ATP in 1x kinase buffer (as above) with or without SB 203580 at 0.5 μM, and with or without active p38β(see as above) (at 50 ng for lane 1 & 3; 100 ng for lane 4; 200 ng for lane 5 & 6. Zero for lane 2 under optimized conditions.

Using the *in vitro* kinase assay of Example 3, the present inventors have observed that all forms of p38 MAPK (alpha, beta, gamma, and delta) phosphorylate both ERα and ERβ and that the p38α- and p38β-induced phosphorylation of ER can be inhibited by the p38 specific inhibitor SB 203580.

EXAMPLE 5

The instant invention provides *in vitro* data showing that p38 MAPK is capable of functionally activating ER not only by direct phosphorylation of ER, but also by phosphorylating the ER coactivator, Amplified in Breast Cancer 1 (AIB1).

Figure 4 shows *in vitro* phosphorylation (assay according to Example 3) of AIB1 by p38 MAPK. Purified (recombinant) p38 MAP kinase phosphorylates Xenopus oocyte-expressed AIB1 (Lanes 3,5) similarly to Extracellular Signal-Regulated Kinase 2 (ERK 2)(lane 7). The p38-specific inhibitor SB 203580 inhibits AIB1 phosphorylation by p38 MAPK (lane 4). Lanes 2 and 8 are positive controls for p38 MAPK and ERK kinase reaction with the known substrates Activating Transcription Factor 2 (ATF2) and ETS-like Transcription Factor-1 (ELK1), respectively.

These results indicate that p38 MAPK is able to phosphorylate ER coactivator AIB1. Therefore, p38 MAPK inhibitors may ameliorate, suppress, prevent or eliminate tamoxifen resistance and/or endocrine resistance, in part, by preventing AIB1 phosphorylation and , in part, by blocking p38 MAPK-mediated phosphorylation of ER.

EXAMPLE 6

According to this example, high AIB1 correlated with a reduction in disease-free survival in patients receiving tamoxifen, indicative of tamoxifen resistance. AIB1 is activated by ERK1,2 MAPKs. When expression of AIB1 and HER-2, which activates ERK1,2 MAPKs, are considered together, only those patients whose tumors contained both high AIB1 and high HER-2 are resistant to tamoxifen. The antitumor activity of tamoxifen in patients with breast cancer is thus determined, in part, by tumor levels of AIB1, so that AIB1 may be an important new diagnostic and therapeutic target. *See also* WO 00/34788. In a similar way, the phosphorylation of AIB1 by p38 MAPK may have the same clinical importance and consequences.

The ER coactivator AIB1 is often amplified and overexpressed in breast tumors, and we have recently found that AIB1 is an important component of TamR found in Her2-overexpressing tumors. Interestingly, we found that both JNK and p38 MAPK can directly phosphorylate AIB1 *in vitro*. Thus, as has been suggested for growth factor signaling, AIB1 may also be a conduit for kinase-mediated stress signaling to the ER pathway. Our data suggests that increased active JNK and p38, and cross-talk between these pathways and the ER pathway, may play a key role in endocrine resistance through phosphorylation and activation of different components of the ER pathway. We are currently studying whether specific JNK and p38 inhibitors can circumvent endocrine resistance *in vivo* in our xenograft breast cancer model.

REFERENCES

The documents cited throughout this application are incorporated herein in their entirety by reference. Citation of these documents is not to be construed as an admission that such documents are available as "prior art" against the instant invention.

CLAIMS

We claim:

1. A method of diagnosing a tamoxifen-resistant breast or prostate tumor in an individual comprising:

providing a biological sample from the individual, and

detecting p38 MAPK activity or expression within said sample,

wherein higher p38 MAPK activity or expression in said biological sample compared to a sample obtained from a control tumor known to be tamoxifen-sensitive indicates that said biological sample is more tamoxifen resistant than the control tumor.

2. The method of claim 1 wherein the biological sample is derived from a tissue biopsy.

3. The method of claim 1 wherein the biological sample is obtained from a tumor.

4. The method of claim 1 wherein said detecting comprises detecting p38 MAPK activity.

5. The method of claim 4 wherein p38 MAPK activity is detected by a phosphorylation assay selected from the group consisting of an in-gel kinase assay, an immunohistochemical assay, an immunofluorescence assay, a Western blot assay, and an ELISA.

6. The method of claim 1 wherein said detecting comprises detecting p38 MAPK expression.

7. The method of claim 6 wherein p38 MAPK expression is detected by nucleic acid hybridization.

8. The method of claim 6 wherein p38 MAPK expression is detected using an expression assay selected from the group consisting of a PCR assay, a Northern blotting assay, an *in situ* hybridization assay, a nuclear run-on assay, a reporter gene assay, an immunohistochemical assay, an immunofluorescence assay, a Western blot assay, and an ELISA.

9. A method of diagnosing an estrogen withdrawal-resistant breast or prostate tumor in an individual comprising:

providing a biological sample from the individual, and

detecting p38 MAPK activity or expression within said sample,

wherein higher p38 MAPK activity or expression in said biological sample compared to a sample obtained from a control tumor known to be estrogen withdrawal-sensitive indicates that said biological sample is more estrogen withdrawal-resistant that the control tumor.

10. The method of claim 9 wherein the biological sample is derived from a tissue biopsy.

11. The method of claim 9 wherein the biological sample is obtained from a tumor.

12. The method of claim 9 wherein said detecting comprises detecting p38 MAPK activity.

13. The method of claim 12 wherein p38 MAPK activity is detected by a phosphorylation assay selected from the group consisting of an in-gel kinase assay, an immunohistochemical assay, an immunofluorescence assay, a Western blot assay, and an ELISA.

14. The method of claim 9 wherein said detecting comprises detecting p38 MAPK expression.

15. The method of claim 14 wherein p38 MAPK expression is detected by nucleic acid hybridization.

16. The method of claim 14 wherein p38 MAPK expression is detected using an expression assay selected from the group consisting of a PCR assay, a Northern blotting assay, an *in situ* hybridization assay, a nuclear run-on assay, a reporter gene assay, an immunohistochemical assay, an immunofluorescence assay, a Western blot assay, and an ELISA.

17. A method of identifying a molecule that prevents or reverses acquired tamoxifen resistance in a breast or prostate tumor comprising:

contacting a tamoxifen-resistant tumor cell with a test molecule; and

detecting p38 MAPK activity or expression within said sample;

wherein lower p38 MAPK activity or expression in said tumor cell upon or after contact with the test molecule compared to the p38 MAPK activity or expression before contact with the test molecule indicates that the agent is likely to prevent or reverse acquired tamoxifen resistance in a tumor.

18. The method of claim 17 wherein said tumor cell is a breast tumor cell.

19. The method of claim 17 wherein said tumor cell is transfected with a nucleic acid comprising a p38 MAPK expression control sequence operably linked to a reporter gene.

20. A method of identifying a molecule that prevents or reverses acquired estrogen withdrawal resistance in a breast or prostate tumor comprising:

contacting an estrogen withdrawal-resistant tumor cell with a test molecule; and

detecting p38 MAPK activity or expression within said sample;

wherein lower p38 MAPK activity or expression in said tumor cell upon or after contact with the test molecule compared to the p38 MAPK activity or expression before contact with the test molecule indicates that the agent is likely to prevent or reverse acquired estrogen withdrawal resistance in the tumor.

21. The method of claim 20 wherein said tumor cell is a breast tumor cell.

22. The method of claim 20 wherein said tumor cell is transfected with a nucleic acid comprising a p38 MAPK expression control sequence operably linked to a reporter gene.

23. A method of reversing or preventing tamoxifen resistance in a tumor comprising:

contacting said tumor with a p38 MAPK inhibitor in an amount effective to inhibit p38 MAPK.

24. The method of claim 23 wherein said tumor is a breast tumor.

25. The method of claim 23 wherein said tumor is a human tumor.

26. The method of claim 23 wherein said tamoxifen resistance is acquired tamoxifen resistance.

27. The method of claim 23 wherein said p38 MAPK inhibitor is selected from the group consisting of antisense p38 MAPK nucleic acids and fragments thereof and anti-p38 MAPK antibodies and fragments thereof.

28. The method of claim 23 wherein said p38 MAPK inhibitor is selected from the group consisting of EO-1428, PD169316, SB202190, SB203580, SB239063, SB281832, VX-702, VX-745, ZM336372, RPR 200765A, and N-(3-tert-butyl-1-methyl-5-pyrazolyl)-N'-(4-(4-pyridinylmethyl)phenyl)urea.

29. A method of reducing, reversing or preventing endocrine resistance in a tumor comprising:

contacting said tumor with a p38 MAPK inhibitor in an amount effective to inhibit p38 MAPK.

30. The method of claim 29 wherein said tumor is a breast tumor.

31. The method of claim 29 wherein said said tumor is a human tumor.

32. The method of claim 29 wherein said endocrine resistance is *de novo* endocrine resistance.

33. The method of claim 29 wherein said endocrine resistance is acquired endocrine resistance.

34. The method of claim 29 wherein said endocrine resistance is tamoxifen resistance.

35. The method of claim 29 wherein said endocrine resistance is estrogen withdrawal resistance.

36. The method of claim 29 wherein said p38 MAPK inhibitor is selected from the group consisting of antisense p38 MAPK nucleic acids and fragments thereof and anti-p38 MAPK antibodies and fragments thereof.

37. The method of claim 29 wherein said p38 MAPK inhibitor is selected from the group consisting of EO-1428, PD169316, SB202190, SB203580, SB239063, SB281832, VX-702, VX-745, ZM336372, RPR 200765A, and N-(3-tert-butyl-1-methyl-5-pyrazolyl)-N'-(4-(4-pyridinylmethyl)phenyl)urea.

38. A method of increasing the tamoxifen sensitivity of tamoxifen-resistant tumor comprising:

contacting said tumor with a p38 MAPK inhibitor in an amount effective to inhibit p38 MAPK.

39. The method of claim 38 further comprising contacting said tumor with tamoxifen.

40. The method of claim 38 wherein said p38 MAPK inhibitor is selected from the group consisting of antisense p38 MAPK nucleic acids and fragments thereof and anti-p38 MAPK antibodies and fragments thereof.

41. The method of claim 38 wherein said p38 MAPK inhibitor is selected from the group consisting of EO-1428, PD169316, SB202190, SB203580, SB239063, SB281832, VX-702, VX-745, ZM336372, RPR 200765A, and N-(3-tert-butyl-1-methyl-5-pyrazolyl)-N'-(4-(4-pyridinylmethyl)phenyl)urea.

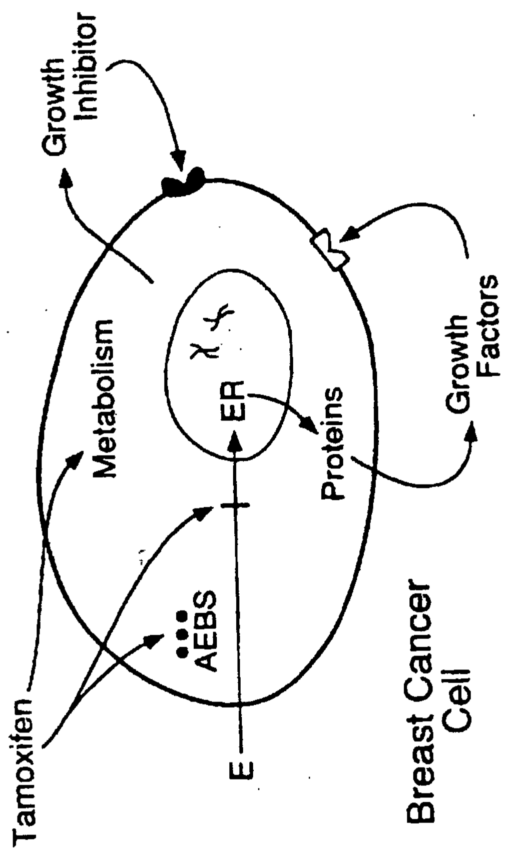
Growth Inhibitor
Growth
Metabolism
Tamoxifen
AEBS
E
ER
Proteins
Growth Factors
Breast Cancer Cell

Fig. 1

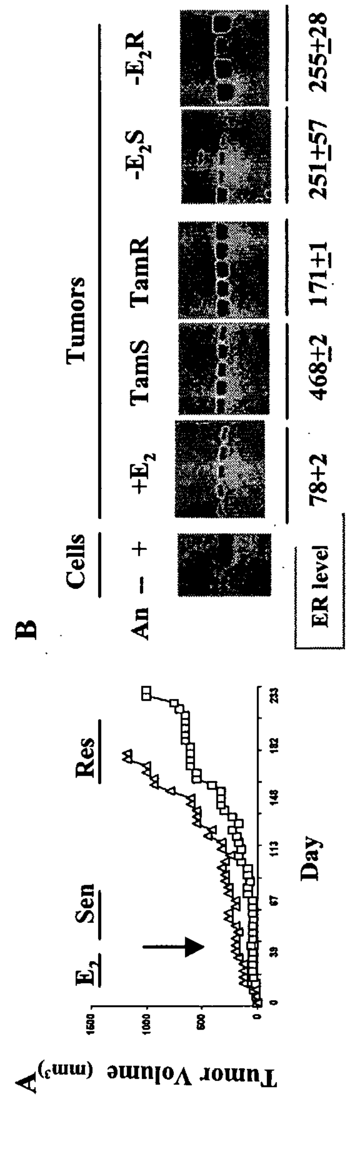
A
Tumor Volume (mm³)
Day
E2
Sen
Res
B
Cells
Tumors
An − +
+E2
TamS
TamR
-E2S
-E2R
ER level
78+2
468+2
171+1
251+57
255+28

Fig. 2

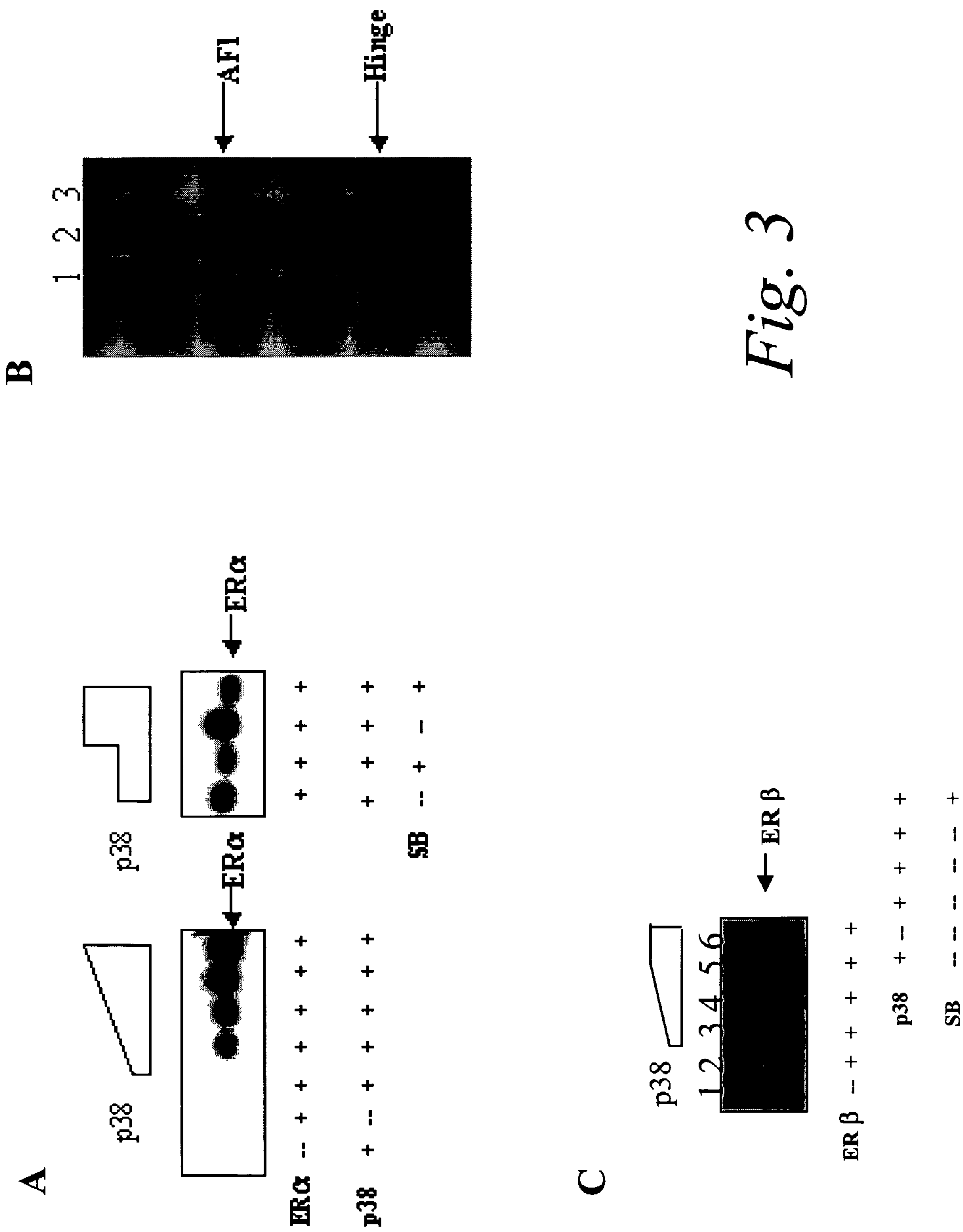

Fig. 3

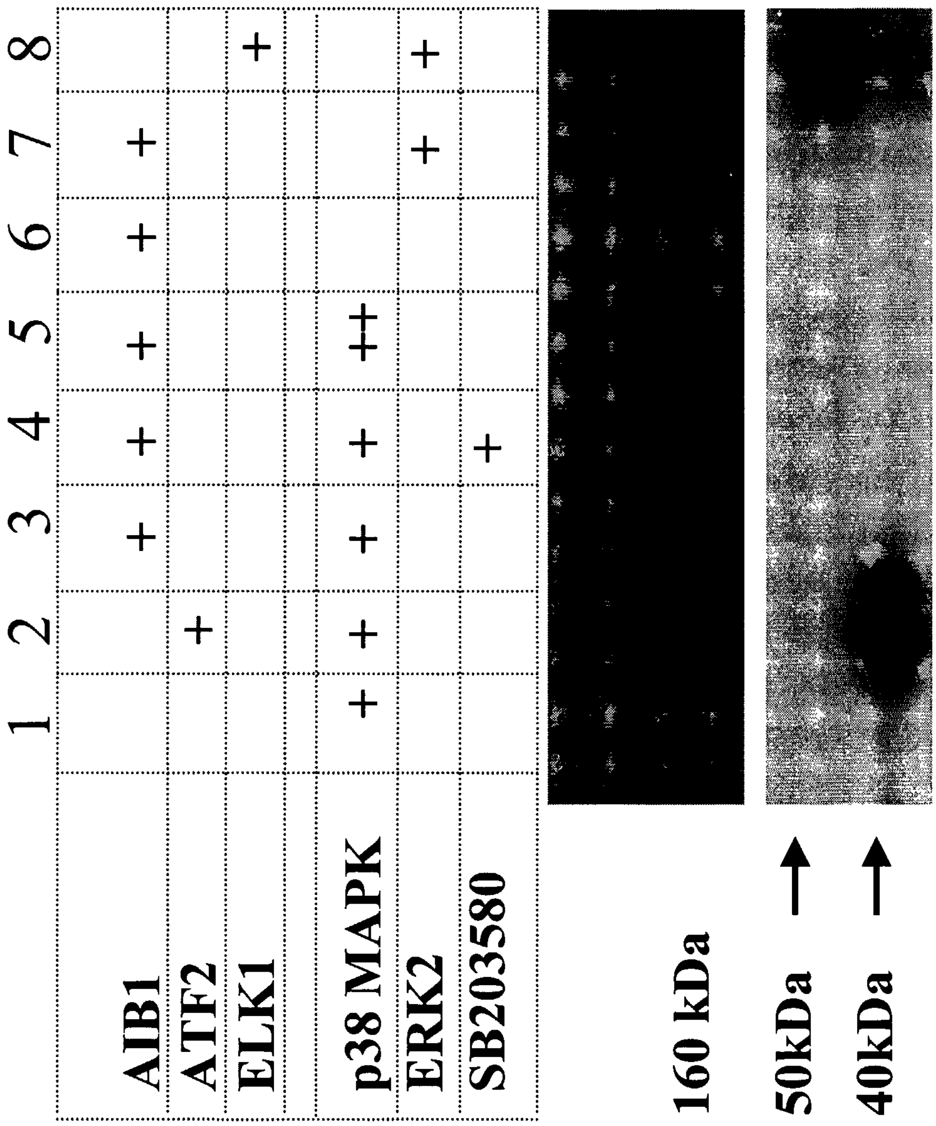
1
2
3
4
5
6
7
8
AIB1
ATF2
ELK1
p38 MAPK
ERK2
SB203580
160 kDa
50kDa
40kDa

Fig. 4