Microtubule-targeted chemotherapeutic agents

inhibit STAT3 signaling

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Abbreviations: Signal transducer and activator of transcription (STAT), Interferon (IFN), Janus Kinase (Jak), interleukin-6 (IL-6), Leukemia inhibitory factor (LIF), Nuclear factor-kB (NFκB), suppressors of cytokine signaling (SOCS), protein inhibitors of STATs (PIAS)

Abstract

The transcription factor STAT3 is inappropriately activated in the majority of breast tumors, especially in aggressive and invasive ones. In addition to driving expression of genes promoting malignancy, STAT3 associates with tubulin and can promote cell migration. Since microtubule targeted drugs are among the most active agents used in the treatment of breast cancer, we examined whether microtubule based chemotherapy modulates STAT3 activity. When treated with paclitaxel or vinorelbine, breast cancer cells with constitutive activation of STAT3 display a loss of STAT3 phosphorylation, and paclitaxel disrupts the interaction of STAT3 with tubulin. Paclitaxel also inhibits cytokine-induced STAT3 activation. This effect is specific for microtubule targeted agents, as other chemotherapeutic drugs, such as doxorubicin, have no effect on STAT3. The loss of STAT3 tyrosine phosphorylation is also reflected in an inhibition of expression of STAT3 target genes. This effect is not restricted to breast cancer, as similar effects are also seen in ovarian cancer and prostate cancer cells. Thus, in addition to their role in disrupting microtubule function, microtubule-targeted agents also suppress STAT3 signaling. This may be an important component of their activity, raising the possibility that microtubule targeted therapy may be particularly effective in tumors characterized by STAT3 activation.

Introduction

Breast cancer is the second leading cause of cancer-related deaths in women in the United States, and often arises due to aberrant activation of transcription factors that play important roles in normal mammary function (Visvader and Lindeman, 2003). Important among these are the signal transducers and activators of transcription (STAT) family of transcription factors.

STATs are latent transcription factors that reside in the cytoplasm until activated by tyrosine phosphorylation. STATs can be phosphorylated via the JAK family of kinases as well as by other receptor and non-receptor tyrosine kinases. Tyrosine phosphorylated STAT dimers translocate to the nucleus, bind DNA, and modulate transcription of target genes involved in many cellular processes. In normal cells, STAT signaling is rapid and transient; in cancer cells, by contrast, STATs are often activated constitutively, leading to enhanced expression of genes promoting proliferation, survival, and invasion. One STAT family member in particular, STAT3, is constitutively activated in a majority of breast cancers. While tyrosine phosphorylation is critical for STAT3 function, STAT3 can also be phosphorylated on a specific serine residue, ser727. STAT3 serine phosphorylation may enhance STAT3-mediated transcription (Zhang et al., 1995), but it can also inhibit the tyrosine phosphorylation of STAT3 (Chung et al., 1997). In normal cells, after modulating gene expression, STATs become dephosphorylated by tyrosine phosphatases and are thus free for subsequent rounds of stimulation (Chen et al., 2003; David et al., 1993; Haque et al., 1995). STAT signaling is also inhibited via two additional pathways: SOCS family members are STAT target genes that bind to receptors and block further STAT activation, thereby turning off the

initial signal (Denson et al., 2003; Galm et al., 2003; Morita et al., 2000; Tonko-Geymayer et al., 2002), and PIAS proteins inhibit STATs through direct interaction (Chung et al., 1997; Liu et al., 1998). Many of these inhibitory pathways are inactivated in cancer cells, resulting in constitutively activated STATs.

In addition to the canonical role of STATs in regulating transcription, STAT3 has other non-transcription based roles. Tyrosine phosphorylated STAT3 may be located at the leading edge of migrating cells, specifically at focal adhesions, where it promotes migration (Silver et al., 2004). Moreover, both JAKs and STATs can be associated with microtubules (Lopez-Perez and Salazar, 2006; Ma and Sayeski, 2007), and the interaction between STAT3 and microtubules promotes migration by competing with the binding of the microtubule associated protein stathmin (Ng et al., 2006).

STAT3 is activated in 70% of breast tumors and is often associated with aggressive and invasive tumors (Alvarez et al., 2005; Walker et al., 2009). Furthermore, inhibition of STAT3 leads to a reversion of the malignant phenotype of these cells, indicating that it is a key mediator of breast cancer pathogenesis. Elucidating the role of STAT3 in breast cancer, and identifying methods to inhibit STAT3, would be beneficial for developing rational therapies for cancer. Among the most active drugs used in the treatment of breast cancer are microtubule-targeting agents. Two types are utilized: microtubule stabilizers such as paclitaxel (Taxol), and microtubule destabilizers such as vinorelbine (Navelbine). Since STAT3 is activated in the majority of breast cancers and associates with microtubules, we wanted to determine if microtubule-targeted therapy modulates STAT3 signaling and function in breast cancer cells.

Materials and Methods

Cell lines

MDA-MB-468 (ATCC, Manassas, VA), MDA-MB-231 (ATCC), MCF-7 (kindly provided

by Francis Kern, Southern Research Institute, Birmingham, AL), NIH-3T3 STAT1-luc

(Lynch et al., 2007), and U3A STAT3-luc (Lynch et al., 2007) were maintained in DMEM

containing 10% fetal calf serum (FCS). SKOV3 and OVCAR8 cell lines (both kindly

provided by Ronny Drapkin, DFCI), BT549 cells (kindly provided by Kornelia Polyak,

DFCI), and DU145 cells (kindly provided by Max Loda, DFCI) were maintained in RPMI

1640 containing 10% FCS. Cells were treated with paclitaxel, vinorelbine, or

doxorubicin (Novaplus), with 10 mg/ml cycloheximide (Sigma, St. Louis, MO), 20 μM

UO126 (Cell Signaling, Beverly, MA), or with Jak inhibitor 1 at the concentration

indicated (Calbiochem, San Diego, CA), 10 ng/ml leukemia inhibitory factor (LIF)

(Chemicon, Temecula, CA), 10 ng/ml oncostatin M (OSM), 10 ng/ml interleukin 6 (IL-6),

or 500 U/ml interferon gamma (IFNγ) (R & D Systems, Minneapolis, MN).

Immunoblots and immunoprecipitations

Immunoblots were performed as described (Nelson et al., 2008); the following

antibodies were used: phospho-tyrosine-STAT3 (#9131), phospho-akt (#9271), akt

(#9272), phospho-src (#2101), phospho-p44/42 MAPK (#9101), p44/42 MAPK (#9102),

all from Cell Signaling; phospho-serine STAT3 (Frank et al., 1997), STAT3 (sc-482), src

(sc-18) from Santa Cruz Biotechnology (Santa Cruz, CA), and α-tubulin (T-5168, from

Sigma). Immunoprecipitations were performed as described (Nelson et al., 2008) using

anti-Flag antibodies (Sigma), or using NP-40 buffer (1% NP-40 alternative, 0.15 M

NaCl, 0.01 M sodium phosphate, 10% glycerol, 2.5 mM EDTA) with α-tubulin

antibodies. Following immunoprecipitation, immunoblots were performed with anti-

STAT3 antibodies. The density of the bands was quantified by ImageJ (National

Institutes of Health, Bethesda, MD). Error bars represent the standard error of the

mean of at least two different experiments. P values were generated using Student's t

test.

mRNA quantitation

RNA was harvested using the RNeasy Mini kit from Qiagen (Valencia, CA), according to

the manufacturer's protocol. cDNA was generated using a TaqMan first strand kit from

Applied Biosystems (Foster City, CA), and qPCR was performed in triplicate using

SYBR green master mix (Applied Biosystems) on a 7500 Real Time PCR system

(Applied Biosystems) with the following parameters: 95 °C for 10 minutes followed by 40

cycles of 95°C for 15s and 60°C for 60s, and then a dissociation stage. Data are

expressed as mean fold change +/- s.e.m. Primer sequences are provided in

Supplemental Table 1.

Luciferase reporter assays

MDA-MB-468 cells (5 X 10^4 cells) were transfected in duplicate with 1 μg of a STAT-

responsive firefly luciferase reporter plasmid (M67-luc, kindly provided by J. Bromberg,

Memorial Sloan-Kettering, New York, NY) or with NFκB-responsive NFκB-luc

(Stratagene) using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were also

transfected with 0.1 μg of a plasmid that constitutively expresses Renilla luciferase

(phRL-TK, Promega, Madison, WI) as a transfection control. Six hours after

transfection, medium was replaced and drug or vehicle was added. Luciferase activity

was quantitated 22 hours later as described (Walker et al., 2007). To analyze the

specificity of paclitaxel on particular STAT family members, cell lines that stably express

the M67-luc construct were used in which luciferase expression is driven by a specific

STAT in isolation. STAT1 (and not STAT3) is activated in response to IFNγ in NIH-3T3-

luc cells therefore, only STAT1 activity is measured (Lynch et al., 2007). In U3A-luc

fibrosarcoma cells, STAT1 is not expressed, and therefore, STAT3 is the only STAT

activated in response to IL-6 (Lynch et al., 2007). Cells were pretreated with 7 μM

paclitaxel for one hour followed by stimulation with IFNγ (STAT1 NIH3T3-luc cells) or IL-

6 (STAT3 U3A-luc cells) for 6 hours. Data are expressed as mean +/- standard

deviation and are representative of at least two different experiments.

Viability measurement

Viability was assayed in MDA-MB-468 and MDA-MB-231 cells (5 X10^3) and DU145 cells

(3 X10^3), plated in duplicate in 96 well dishes. Twenty-four hours after plating, drug was

added at the indicated doses. Viability was measured 48 hours after drug treatment by

ATP-dependent bioluminescence (Celltiter-Glo, Promega). All values were normalized

to the vehicle treated. Each data point is representative of at least two different

experiments.

Results

Microtubule based chemotherapy inhibits STAT3 tyrosine phosphorylation

To determine the effect of microtubule-targeted agents on STAT3 signaling, we utilized MDA-MB-468 cells, which contain high levels of phosphorylated STAT3, and are sensitive to paclitaxel treatment (Supplemental figure 1). Treatment with paclitaxel resulted in a dose- and time-dependent decrease in tyrosine phosphorylated STAT3 in MDA-MB-468 cells (Figure 1a and b). Paclitaxel also inhibited STAT3 tyrosine phosphorylation in BT549 cells, another breast cancer cell line containing constitutively active STAT3 (Supplemental Figure 2).

To determine if paclitaxel only inhibits constitutively active STAT3, we utilized MCF-7 cells which do not contain phosphorylated STAT3, but in which STAT3 becomes activated in response to leukemia inhibitory factor (LIF). In cells pretreated with paclitaxel, the induction of STAT3 phosphorylation was reduced by 26% (Supplemental Figure 3). Similar results were obtained in mouse embryonic fibroblasts activated by LIF (data not shown). Therefore, paclitaxel not only inhibits STAT3 in breast cancer cells that contain constitutively active STAT3, but paclitaxel also inhibits the induction of STAT3 phosphorylation.

Inhibition of STAT3 phosphorylation was not restricted to breast cancer cells, as paclitaxel also inhibited STAT3 phosphorylation in the ovarian cancer cell lines OVCAR8 and SKOV3 (Supplemental Figure 4), and the prostate cancer cell line DU145 (Supplemental Figure 5). The inhibition of STAT3 tyrosine phosphorylation did not arise from cytotoxicity, as any changes in cellular viability occurred much later then changes in STAT3 tyrosine phosphorylation (data not shown).

To determine if paclitaxel inhibited STAT3 specifically, or if paclitaxel affected

multiple pathways, signaling molecules known to be activated in breast cancer cells

were analyzed. Paclitaxel treatment of MDA-MB-468 cells inhibited STAT3 tyrosine

phosphorylation (by 30% in this experiment), but did not inhibit the phosphorylation of

SRC, MAP kinase, or AKT (Figure 1c). This suggests that the inhibition of STAT3 by

paclitaxel is not due to global inhibition of cellular signaling.

To determine if STAT3 inhibition was specific to microtubule stabilizers such as

paclitaxel or if it was due to modulation of microtubule function in general, we next

examined the effect of an equitoxic dose of the microtubule destabilizer vinorelbine

(Navelbine) which is also an active agent in breast cancer therapy. Similar to paclitaxel,

vinorelbine decreased STAT3 tyrosine phosphorylation by approximately 50% in MDA-

MB-468 cells (Figure 1d). Thus, STAT3 tyrosine phosphorylation is inhibited by both

microtubule stabilizers as well as destabilizers. To exclude the possibility that all

cytotoxic agents lead to a loss of STAT3 function, we treated MDA-MB-468 cells with an

equitoxic dose of doxorubicin, a DNA intercalating agent and topoisomerase II inhibitor,

which is a potent chemotherapy drug used in the treatment of breast cancer

(Supplemental Figure 6). Treatment of MDA-MB-468 cells with doxorubicin at equitoxic

doses had no effect on STAT3 tyrosine phosphorylation (Figure 1d). These results

indicate that the loss of STAT3 tyrosine phosphorylation is not a non-specific effect of

chemotherapy drug treatment but is specific to microtubule targeted therapy.

Paclitaxel inhibits STAT3 dependent reporter gene expression

Given that paclitaxel decreases STAT3 phosphorylation, we next considered whether paclitaxel inhibited STAT3-dependent gene regulation. We first examined how paclitaxel affected the expression of a luciferase reporter gene driven by a STAT-dependent promoter. MDA-MB-468 cells contain constitutive activation of STAT3 but not STAT1 (data not shown), so the STAT-dependent M67-luc reporter construct used only measures STAT3 activity in these cells. MDA-MB-468 cells were transfected with M67-luc and then treated with paclitaxel. STAT3-dependent luciferase expression was reduced by 40-60% in paclitaxel-treated cells compared to those treated with vehicle control (Figure 2a). The reduction in levels of luciferase was similar to that following treatment with Jak inhibitor 1 (Figure 2a), which inhibits STAT3 tyrosine phosphorylation in these cells (data not shown). To determine if this effect was specific to STAT3 or if paclitaxel also influences other transcription factors, an NFκB-dependent reporter was transfected into MDA-MB-468 cells, which also have high levels of constitutive NFκB activity (Newton et al., 1999). In contrast to its reduction of STAT3-dependent luciferase activity, paclitaxel treatment had no effect on NFκB -dependent luciferase activity (Figure 2a); this indicates that paclitaxel specifically inhibits STAT3- but not NFκB-dependent gene expression.

To determine if paclitaxel also inhibits cytokine-induced STAT3-dependent reporter expression, we used U3A cells that were engineered to stably express a STAT3-dependent luciferase construct. Cells were pretreated with paclitaxel and then stimulated with IL-6 to activate STAT3. IL-6 stimulation resulted in a prominent increase of luciferase expression in cells pretreated with vehicle; however, following paclitaxel pretreatment, the induction by IL-6 was decreased by approximately 50% (Figure 2b).

Similar results were also obtained in mouse embryonic fibroblasts (MEFs) transfected with a STAT3-dependent luciferase reporter gene (data not shown), indicating that paclitaxel inhibits both constitutive and cytokine-induced STAT3-dependent gene expression. To determine if this effect of paclitaxel is specific to STAT3 or whether all STATs are similarly affected, NIH-3T3 cells stably expressing a STAT1-dependent luciferase construct were stimulated with IFNγ, which activates STAT1. Compared to vehicle, paclitaxel pretreatment had no effect on IFNγ induction of luciferase (Figure 2b), indicating that paclitaxel specifically targets STAT3-dependent gene regulation.

Paclitaxel inhibits endogenous STAT3-dependent gene expression

Given that paclitaxel specifically inhibited STAT3-dependent reporter gene expression, we next examined whether this drug also inhibits endogenous STAT3 target genes. MDA-MB-468 cells were treated with paclitaxel or vinorelbine for 6 hours and mRNA expression was analyzed by quantitative RT-PCR for *BCL6*, *BCL3*, and *SMAD7*, all of which are known to be STAT3 target genes. Paclitaxel and vinorelbine treatment inhibited the expression of each of these STAT3 target genes by 80-90% (Figure 2c), indicating that paclitaxel and vinorelbine functionally inhibit STAT3 signaling.

Paclitaxel induces STAT3 serine phosphorylation

It has been reported that inhibition of STAT3 tyrosine phosphorylation can be mediated by increased phosphorylation of STAT3 on serine 727 (Chung et al., 1997). We thus considered the possibility that paclitaxel and vinorelbine inhibit STAT3 tyrosine phosphorylation through induction of STAT3 serine phosphorylation. Treatment of

MDA-MB-468 cells with paclitaxel (Figure 3a) or vinorelbine (data not shown) enhanced

STAT3 serine phosphorylation, concurrent with the loss of STAT3 tyrosine

phosphorylation. Given this finding, we next tested the possibility that serine

phosphorylation is necessary to mediate the inhibition of tyrosine phosphorylation. A

mutant form of STAT3 in which serine 727 has been changed to alanine (S->A) was

stably expressed in MDA-MB-468 cells; these cells were treated with paclitaxel,

followed by immunoprecipitation and immunoblotting. The S->A mutant of STAT3 had

no effect on the inhibition of STAT3 tyrosine phosphorylation by paclitaxel (Figure 3b),

demonstrating that induction of serine phosphorylation is not a prerequisite for the

inhibition of STAT3 tyrosine phosphorylation by paclitaxel.

Paclitaxel inhibition of STAT3 tyrosine phosphorylation requires protein synthesis

Since the inhibition of STAT3 tyrosine phosphorylation by paclitaxel was not an

immediate effect (Figure 1b), we considered the possibility that this inhibition requires

gene transcription and translation. To assess this, MDA-MB-468 cells were treated with

cycloheximide to prevent protein synthesis, prior to treatment with paclitaxel.

Cycloheximide treatment prevented the inhibition of STAT3 tyrosine phosphorylation by

paclitaxel, but did not affect serine phosphorylation of STAT3 (Figure 3c), showing a

specific need for protein synthesis in order for paclitaxel to inhibit STAT3 signaling.

Given this finding, we hypothesized that paclitaxel induces the expression of a

negative regulator of STAT3 signaling. However, paclitaxel did not significantly

upregulate the mRNA expression of any of the five phosphatases (PP2A, PTP1B,

PTP1C, SHP1 and SHP2), eight SOCS family members (SOCS1, SOCS2, SOCS3,

SOCS4, SOCS5, SOCS6, SOCS7, and CIS), or four PIAS family members (PIAS1,

PIAS2, PIAS3, and PIAS4) analyzed (Supplemental Figure 7). Therefore, the inhibition

of STAT3 signaling induced by paclitaxel does not appear to be mediated through

increased expression of one of these negative regulators.

Paclitaxel inhibits the association of STAT3 with tubulin

Since STAT3 has been reported to interact directly with tubulin, we considered

the possibility that paclitaxel was inhibiting STAT3 function by interfering with its

association with this protein. MDA-MB-468 cells were treated with vehicle, paclitaxel, or

Jak inhibitor 1 and STAT3 phosphorylation was analyzed. Both paclitaxel and Jak

inhibitor 1 inhibited STAT3 tyrosine phosphorylation (Figure 3d). Immunoprecipitation

was then performed with antibodies to tubulin, and associated STAT3 was determined

by immunoblot. The association of STAT3 with tubulin was lost upon paclitaxel

treatment (Figure 3d); however, inhibiting STAT3 tyrosine phosphorylation with Jak

Inhibitor 1 did not modify the association between STAT3 and tubulin. Thus, paclitaxel

disrupts the association of STAT3 with tubulin, but does this independent of an effect on

STAT3 phosphorylation.

Inhibition of STAT3 phosphorylation by paclitaxel correlates with cytotoxic effect

To determine if the inhibition of STAT3 tyrosine phosphorylation by paclitaxel and

vinorelbine correlate with the cytotoxic effects of these drugs, we analyzed the

sensitivity of additional breast cancer cell lines to these agents. MDA-MB-231 cells are

less sensitive to the cytotoxic effects of paclitaxel and vinorelbine than MDA-MB-468

cells (Figure 4a and Supplemental Figure 8). Similarly, neither microtubule targeted

therapy was effective in inhibiting STAT3 tyrosine phosphorylation, or inducing STAT3

serine phosphorylation (Figure 4b). Furthermore, paclitaxel had no effect on the

interaction of STAT3 with tubulin in MDA-MB-231 cells (Figure 4c). These findings

suggest that the cytotoxic effects of paclitaxel and vinorelbine correlate with the ability of

these drugs to inhibit STAT3 tyrosine phosphorylation.

Discussion

Microtubule-targeted chemotherapeutic drugs are among the most active agents

in the treatment of breast cancer. We have shown that microtubule stabilizers as well

as destabilizers inhibit STAT3 signaling, which results in loss of STAT3 tyrosine

phosphorylation and function. These drugs also disrupt the interaction of STAT3 with

tubulin, thereby further blocking STAT3 function.

Both paclitaxel and vinorelbine inhibit STAT3 signaling, and this ability correlates

with the cytotoxic effects of these agents (Figure 4). This raises the possibility that

inhibition of STAT3 is an important event in the cell death induced by these agents. To

address this, we had hoped to rescue cells with a constitutively active STAT3 mutant

(Bromberg et al., 1999); however, microtubule-targeted agents decrease the tyrosine

phosphorylation that is necessary for the activity of this mutant (Liddle et al., 2006), and

thus we were unable to assess this possibility (data not shown). Through their effects

on microtubules, drugs like paclitaxel can undoubtedly kill cells that lack STAT3

activation. However, the results presented here suggest that inhibition of STAT3 may

be an important component of the effects of microtubule targeted therapy on tumor cells that contain activated STAT3.

Several mechanistic questions remain as to how the microtubule targeting agents inhibit STAT3 activation. Although we examined 17 potential negative regulators of STAT3 signaling, we were not able to identify one whose expression is increased significantly following treatment with paclitaxel. However, analysis of signaling pathways and transcription factor activation upon paclitaxel treatment may provide insight into the mechanism of paclitaxel-induced STAT3 inhibition. We did find that the interaction between STAT3 and microtubules was disrupted by paclitaxel, and this may be an important component of the effects of microtubule targeting agents on this pathway. Paclitaxel may also function on non-phosphorylated forms of STAT3, thereby preventing their translocation to the nucleus (Lopez-Perez and Salazar, 2006), or it may prevent their binding to focal adhesions (Silver et al., 2004), thus, inhibiting many forms of STAT3 signaling.

In conclusion, microtubule-targeted therapies specifically target STAT3 signaling via inhibition of STAT3 tyrosine phosphorylation and loss of interaction of STAT3 with tubulin. Since STAT3 activation is associated with high-grade cancers, targeting STAT3 is a potentially important approach to the therapy of breast cancer. These results suggest that screening for the STAT3 activation status of tumors may help to identify those patients most likely to benefit from microtubule-targeted therapies. In addition, a number of STAT3 inhibitors have recently been identified (Nelson et al., 2008; Song et al., 2005; Turkson et al., 2004). This raises the possibility that the combination of a novel STAT3 inhibitor with a microtubule-targeted therapy will work in synergy to

provide a better therapeutic effect, with fewer side effects, in breast cancers with

activated STAT3.

Conflict of Interest

The authors have no conflicts to disclose.

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Footnotes

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Figure legends

Figure 1. Paclitaxel inhibits STAT3 tyrosine phosphorylation. Tyrosine phosphorylated and total STAT3 was analyzed by immunoblot in MDA-MB-468 cells treated with paclitaxel at the indicated doses for 6 hours (A), or treated with 7 μM paclitaxel for the indicated times (B). MDA-MB-468 cells were treated with 7 μM paclitaxel for 6 hours and analyzed by immunoblot for the total and phosphorylated forms of the indicated signaling molecules (C). MDA-MB-468 cells were treated with 7 μM paclitaxel, 9.26 μM vinorelbine, or 5 μM doxorubicin for 6 hours and phosphorylated and total STAT3 was analyzed by immunoblot (D). Where indicated, the density of the bands was quantitated from at least two experiments, and the ratio of phosphorylated STAT3 to total STAT3 is shown. Error bars represent the standard error of the mean. * p value < 0.05.

Figure 2. Paclitaxel inhibits STAT3 dependent gene expression. (A) MDA-MB-468 cells were transfected with either a STAT-dependent luciferase reporter gene or an NFκB-dependent luciferase reporter. Cells were then treated with the indicated doses of paclitaxel (in μM) or 0.1 μM Jak inhibitor 1 for 22 hours, and normalized luciferase activity was measured. (B) STAT3-responsive U3A-STAT3-luc and STAT1-responsive NIH-3T3-STAT1-luc cells were pretreated with vehicle or 7 μM paclitaxel for 1 hour followed by stimulation with IL-6 or IFNγ, respectively, for 6 hours and luciferase activity was measured. (C) MDA-MB-468 cells were treated with paclitaxel or vinorelbine for 6 hours and analyzed for expression of the indicated STAT3 target genes (normalized to β-actin).

Figure 3. Paclitaxel induces serine phosphorylation of STAT3 and prevents the interaction of STAT3 with tubulin. (A) MDA-MB-468 cells were treated with 7 µM paclitaxel for 6 hours and analyzed by immunoblot for tyrosine and serine phosphorylation of STAT3. The density of the bands was quantitated and the ratio of phosphorylated to total STAT3 is shown. Error bars represent the standard error of the mean for at least two experiments. (B) MDA-MB-468 cells stably expressing epitope (Flag) tagged constructs of STAT3 were treated with 7 µM paclitaxel for 6 hours, followed by anti-Flag immunoprecipitation. Immunoblots were then performed to tyrosine phosphorylated STAT3 and total (Flag) STAT3. The density of the bands was quantitated and tyrosine phosphorylated STAT3 was compared to total (Flag) STAT3. (C) Paclitaxel inhibition of STAT3 is dependent on protein synthesis. MDA-MB-468 cells were pretreated with vehicle or cycloheximide for 1 hour followed by treatment with paclitaxel for 5 hours. Immunoblots were then performed with the indicated antibodies. The density of the bands was quantitated and tyrosine phosphorylated STAT3 was compared to total STAT3 for two separate experiments. (D) Paclitaxel disrupts the interaction between STAT3 and tubulin. MDA-MB-468 cells were treated with 7 µM paclitaxel or 1 µM JAK Inhibitor 1 for 5 hours, followed by immunoprecipitation with antibodies to STAT3 or tubulin. Immunoblots were performed with the indicated antibodies. The density of the bands was quantitated and STAT3 was normalized to tubulin, as a loading control.

Figure 4. Inhibition of STAT3 phosphorylation correlates with sensitivity of cells to paclitaxel cytotoxicity. (A) MDA-MB-468 and MDA-MB-231 cells were treated with 7 μM paclitaxel for 48 hours and relative viability was measured using ATP-dependent bioluminescence. (B) MDA-MB-231 cells were treated with paclitaxel and vinorelbine for 6 hours and analyzed by immunoblot with the indicated antibodies. The density of the bands was quantitated and the ratio of tyrosine phosphorylated STAT3 to total STAT3 is shown. Error bars represent the standard error of the mean for at least two experiments (C) MDA-MB-231 cells were treated with paclitaxel for 6 hours after which STAT3 and tubulin were immunoprecipitated and analyzed by immunoblot with the indicated antibodies.

Figure 1.

Figure 2.

a.

b.

c.

Figure 3.

a.

P-tyr-STAT3

P-ser-STAT3

STAT3

Paclitaxel: -   +

b.

IP: Flag

vector   wt STAT3   S->A STAT3

P-STAT3

Flag

Paclitaxel: -   +   -   +   -   +

c.

Vehicle   cycloheximide

P-tyr-STAT3

P-ser-STAT3

STAT3

Paclitaxel: -   +   -   +
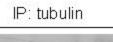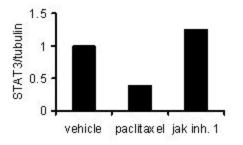
d.

IP: STAT3

P-STAT3

STAT3

vehicle   paclitaxel   jak inh. 1

IP: tubulin

STAT3

tubulin

vehicle   paclitaxel   jak inh. 1

Figure 4.

a.

b.

c.