Anti-mitotic agents such as paclitaxel and docetaxel are widely used for the treatment of breast, ovarian and lung cancers. Although paclitaxel induces apoptosis, this drug also modulates autophagy. How autophagy affects paclitaxel activity, is unclear. We discovered that paclitaxel inhibited autophagy through two distinct mechanisms dependent on cell cycle stage. In mitotic cells, paclitaxel blocked activation of the class III phosphatidyl inositol 3 kinase, Vps34, a critical initiator of autophagosome formation. In non-mitotic paclitaxel-treated cells, autophagosomes were generated but their movement and maturation was inhibited. Chemically or genetically blocking autophagosome formation diminished paclitaxel-induced cell death suggesting that autophagosome accumulation sensitized cells to paclitaxel toxicity. In line with these observations, we identified that primary breast tumors that expressed diminished levels of autophagy-initiating genes were resistant to taxane therapy, identifying possible mechanisms and prognostic markers of clinical chemotherapeutic resistance.

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Keywords: autophagy; paclitaxel; apoptosis

INTRODUCTION
Paclitaxel is the prototypic member of the taxane class of mitotic inhibitor-based chemotherapies. Paclitaxel and its analogs are widely used in the treatment of breast cancer although drug resistance and toxic side effects severely limit taxane effectiveness.3 Paclitaxel binds to β-tubulin, inhibits microtubule depolymerization and arrests cycling cells in M (mitotic) phase.2 Eventually, paclitaxel activates an apoptotic program leading to caspase activation that is regulated by the Bcl-2 family of proteins.3,4

In addition to inducing apoptosis, paclitaxel and analogs also regulate another programmed cell response known as autophagy.5 Autophagy is characterized by the sequestration of cytoplasmic material into double-membrane enclosed vacuoles for degradation by lysosomal hydrolases.6 This process is protective and removes damaged organelles and protein aggregates, yielding free fatty and amino acids for use in cellular metabolism.7 Oppositely, excessive autophagy has been proposed to mediate a caspase-independent cell death program known as programmed cell death type II (PCDII).8 This dual role of autophagy is exemplified by differing cancer treatments where autophagy is protective in response to radiation treatment,7,9,10 but toxic in response to anti-estrogen treatment.11 With respect to taxane treatment of breast cancer cells, it is not clear how paclitaxel regulates autophagy and whether autophagy modulates paclitaxel-induced apoptosis.

In this study, we discovered that paclitaxel inhibited autophagy through two distinct mechanisms dependent on cell cycle stage. In mitotic cells, paclitaxel blocked activation of the class III phosphatidyl inositol 3 kinase, Vps34, a critical initiator of autophagosome formation. In non-mitotic paclitaxel-treated cells, autophagosomes were generated but autophagosome movement was inhibited preventing autophagosome maturation. Further, we showed that by blocking autophagosome formation, paclitaxel-induced apoptosis was diminished, suggesting that autophagy contributed to taxane cytotoxicity. In support of this observation, we found that downregulation of autophagy genes was associated with poor outcome of breast cancer patients. Thus, our data suggest that autophagy enhanced paclitaxel-induced cell death and diminished autophagy may contribute to clinical chemotherapeutic resistance.

RESULTS
Paclitaxel decreases autophagic flux in breast cancer cells
To understand how paclitaxel affects basal autophagy of breast cancer cells, we examined the conversion of the autophagy marker LC3-I to LC3-II in paclitaxel-treated MCF-7 and SK-BR-3 breast carcinoma cell lines (Figure 1a). On autophagic induction, LC3-I is conjugated to phosphatidylethanolamine to form LC3-II. LC3-II localizes to both the inner and outer membranes of the autophagosome and remains until the autophagosomes fuses with the lysosome. At that point, internalized LC3-II is degraded, while the LC3-II molecules on the cytosolic face of the autophagosome can be delipidated and released. As LC3-II levels initially increase, but then decrease as autophagy progresses, we used the lysosomal H+-ATPase inhibitor bafilomycin A1 to differentiate autophagosome formation from turnover and identify the proportion of LC3-II that successfully fused with the lysosome.12,13 Analysis of LC3-II levels in untreated control cells showed an increase in the amount of LC3-II when treated with bafilomycin, indicative of the basal level of autophagy in these cells (Figure 1a, compare lanes 1 and 2). Cells that were deprived of nutrients showed the largest increase in LC3-II with the addition of bafilomycin, demonstrating the greatest autophagic flux (Figure 1a, compare lanes 5 and 6). Paclitaxel-treated cells had showed a decrease in the amount of LC3-II in paclitaxel-treated MCF-7 and SK-BR-3 cells (Figure 1a, compare lanes 4 and 5). Paclitaxel treatment diminished the amount of LC3-II, indicating that autophagy was inhibited in paclitaxel-treated cells.
the lowest levels of LC3-II overall. Overall diminished LC3-II levels may reflect a decrease in autophagosome initiation, as well as an increase in autophagosome degradation. To examine autophagosome degradation directly, cells were treated with both bafilomycin and paclitaxel. Paclitaxel-treated cells showed the lowest level of bafilomycin-dependent increase in LC3-II (Figure 1a, compare lanes 3 and 4) indicating that paclitaxel inhibited the autophagic flow from autophagosome to autophagolysosome. Through analysis of autophagosome dynamics within single cells, we were able to identify cell cycle-associated blocks in autophagy. We generated MCF-7 cell lines that stably expressed green fluorescent protein (GFP)-LC3. Robust levels of basal and

Figure 1. Paclitaxel inhibits accumulation of LC3. (a) MCF-7 cells stably expressing GFP-LC3 were left untreated, treated with 25 nm paclitaxel for the indicated time, or starved in Earle’s balanced salt solution for 4h. Duplicate plates of each condition were treated with 100 nm bafilomycin A1 for the final 4h before harvest. Lysates of each sample were prepared and 18 μg of protein was loaded in each lane of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The gel was then immunoblotted for the indicated proteins. The experiment was performed independently three times and representative blots are shown. (b) MCF-7 cells stably expressing GFP-LC3 were plated on coverslips and left untreated, treated with 25 nm paclitaxel for 24h, or starved in Earle’s balanced salt solution for 4h. Duplicates of each treatment were incubated with 100 nm bafilomycin A1 for the final 4h before fixation. The coverslips were then stained with 4,6-diamidino-2-phenylindole and images were acquired. Representative deconvolved images are shown. Arrowheads denote cells in mitosis. (c) The images in (b) were analyzed using Imaris, and the total number of GFP-LC3 puncta in the non-mitotic cells were counted in each image. For each treatment, the mean number of GFP-LC3 puncta per cell was calculated and the fold induction with bafilomycin A1 treatment was determined. The mean of three independent experiments is displayed as a bar graph with error bars indicating the s.d.
starvation-induced autophagy were demonstrated by increased GFP-LC3 punctate fluorescence in response to bafilomycin treatment (Figure 1b). Paclitaxel treatment resulted in the appearance of two morphologically distinct populations of mitotic and non-mitotic cells. Mitotic cells were identified by chromosome condensation (Figure 1b, middle panel, arrowheads). Mitotic cells showed the least number of GFP-LC3 puncta in both the presence and absence of bafilomycin, indicating that paclitaxel-treated cells in mitotic arrest had diminished autophagosome production and turnover. The non-mitotic paclitaxel-treated cells (Figure 1b, middle panel, arrows) showed an increase in the number and size of GFP-LC3 puncta when compared with untreated control cells, although importantly, bafilomycin treatment did not greatly enhance the number of GFP-LC3 puncta. To quantify the relative autophagic flux in non-mitotic cells in response to paclitaxel treatment, we counted the number of GFP-LC3 puncta per cell and recorded the fold increase in the number of autophagosomes induced by bafilomycin treatment (Figure 1c). This analysis demonstrated that autophagy flux was highest in starved cells (4.5-fold higher relative to non-bafilomycin-treated cells), non-paclitaxel-treated cells showed intermediate turnover (3.5-fold), and paclitaxel-treated cells showed the lowest turnover (1.5-fold) indicating that paclitaxel induced a block in autophagosome maturation. Therefore, paclitaxel inhibited autophagy at two stages: by inhibiting autophagosome formation within mitotically arrested cells, and by inhibiting autophagosome maturation within non-mitotic cells.

Mitotic arrest inhibits autophagosome formation in breast cancer cells
Although paclitaxel induces mitotic arrest, cells can eventually escape and undergo aberrant mitotic exit.4,14,15 This mitotic slippage is required for the demise of certain cell types such as MCF-7 cells.14 Therefore, to monitor autophagic flow with respect to these cell cycle transitions, we analyzed GFP-LC3 fluorescence in single cells over time (Figure 2, two independent time courses are shown as upper and lower). As a cell entered mitotic arrest, GFP-LC3 puncta slowly disappeared and the GFP signal was observed as diffuse fluorescence (Figure 2, arrow, 2–9 h for upper panel and 0–13 h for lower panel). This observation verified that autophagosome formation was blocked in cells in mitotic arrest. As the cell escaped mitotic arrest, GFP-LC3 puncta reappeared (Figure 2, arrowheads, 10–14 h for upper panel and 14–18 h for lower panel), suggesting that inhibition of autophagosome formation was transient and dependent on mitotic arrest. To quantify the proportion of LC3 localized to autophagosomes, we determined the amount of punctate GFP-LC3 fluorescence as a fraction of total GFP-LC3 signal (Figure 2, indicated as % puncta fluorescence). The percentage of punctate GFP-LC3 decreased as the cells entered mitotic arrest, reaching a minimum at 1 h after entry into mitosis and maintained this diffuse fluorescence for the duration of the mitotic arrest (Figure 2, 5–9 h for upper panel and 10–13 h for lower panel). As the cell left mitotic arrest, the percentage of punctate GFP-LC3 increased reaching a 6.5-fold (upper panel) or 3.2-fold (lower panel) increase over the initial percentage, 4 h after exiting arrest (Figure 2, 2 vs 14 h for upper panel and 0 vs 18 h for lower panel). These results suggested that phagophore synthesis and/or progression to LC3-positive structures, was inhibited in response to paclitaxel-induced mitotic arrest, and autophagosome formation was recovered after mitotic slippage.

To better quantitate the autophagic flux in mitotic and non-mitotic cells, we separated the paclitaxel-treated non-adherent cells from the adherent cells. We first verified that the non-adherent cells were in mitotic arrest, as judged by chromosomal morphology. That was indeed the case (Figure 3a). Next, we generated MCF-7 and SK-BR-3 lysates from the mitotic and non-mitotic cell fractions and examined by western blotting for LC3 (Figure 3b). The mitotic cell fraction from both MCF-7 and SK-BR-3 cells showed a small increase in LC3-II band intensity relative to bafilomycin-treated cells, the adherent SK-BR-3 cells showed a greater amount, and the non-paclitaxel-treated cells showed the largest increase, confirming a paclitaxel-dependent inhibition of autophagic flux in SK-BR-3 cells. The MCF-7 cells showed a block in autophagic flux in mitotic cells, with autophagic flux in adherent cells, indicative of cell line differences. The mitotic fraction of both cell lines showed the lowest overall levels of LC3-II, confirming a block in LC3-I to LC3-II conversion. To evaluate whether autophagy progression was indeed inhibited, we examined levels of the autophagic substrate, p62. During successful autophagy, p62 levels decline but bafilomycin treatment results in a rescue of these amounts as can be seen in control MCF-7 and SK-BR-3 cells (Figure 3b, lanes 1 and 2). In the mitotic fraction of both cell lines, bafilomycin-dependent increase in p62 was inhibited (Figure 3b, lanes 5 and 6). Of the adherent cell fractions, MCF-7 cells displayed autophagic degradation of p62, whereas SK-BR-3 showed blunted degradation of p62. These data demonstrated that paclitaxel inhibited overall autophagy progression with mitotically arrested cells showing an early block in autophagosome formation.

Inhibition of autophagosome formation in paclitaxel-mediated mitotic arrest is associated with Vps34 inhibition
As paclitaxel-induced mitotic arrest was associated with diminished formation of LC3-II-positive structures, we tested whether there was a block in autophagosome membrane formation. The first step in autophagosome formation requires the ULK-protein kinase complex and the Vps34 phosphatidyl inositol 3 kinase complex. Furuya et al.16 had shown that during mitosis, Cdk-1 inhibits Vps34 activity through phosphorylation at T159. Therefore, we tested whether paclitaxel-induced inhibitory phosphorylation in Vps34 in mitotic cells. Using an auto-phosho-specific antibody, we found that paclitaxel-treated mitotic cells showed high levels of phosphorylation of Vps34 at T159 in both MCF-7 and SK-BR-3 cells (Figure 4). T159 phosphorylation was diminished in non-mitotic paclitaxel-treated cells. Thus, in mitotic arrest, paclitaxel-mediated block of autophagosome formation was associated with Vps34 inhibition.

Paclitaxel disrupts autophagosome movement
Unlike mitotic cells, paclitaxel-treated non-mitotic cells were strongly positive for LC3-II puncta (Figure 1b). To investigate the dynamics of autophagosome trafficking, we acquired time-lapse images of GFP-LC3 movement in live cells and analyzed puncta speed (Figure 5). When we traced puncta movement over a set period of time, we saw that non-paclitaxel-treated cells showed longer puncta tracks than adherent paclitaxel-treated cells (Figure 5a). We observed that individual puncta movement was highly variable. Thus, the mean speed of movement for each puncta was grouped into low, medium and high speed (Figure 5b). The distribution of the relative classes was significantly altered in paclitaxel-treated cells, with paclitaxel treatment decreasing average puncta speed. The average puncta speed from three independent experiments (Figure 5c) indicated that paclitaxel treatment reduced overall puncta speed to 61% of control levels.

As we observed a decrease in autophagosome speed, we hypothesized that trafficking and autophagosome-to-autophago-lysosome maturation would be affected. In order to examine this possibility, we conducted indirect immunofluorescence staining comparing GFP-LC3 localization with cellular markers (Figure 6). During autophagosome maturation, autophagosomes traffic to the perinuclear region and this can be seen under both normal and starvation conditions, as GFP-LC3-positive puncta localized near the microtubule organizing center marked by pericentrin...
staining (Figure 6, upper). However, in response to paclitaxel treatment, GFP-LC3 puncta localization was dispersed throughout the cell and did not colocalize with pericentrin. Even in the presence of bafilomycin, which elevated perinuclear autophagosome location, GFP-LC3 did not accumulate near the microtubule organizing center in paclitaxel-treated cells. An obvious downstream effect of this loss in autophagosome trafficking would be an inability of autophagosomes to fuse with lysosomes. This was confirmed by comparing localization of GFP-LC3 puncta relative to lysosomes stained with LAMP1. Autophagosomes co-associated with lysosomes in non-paclitaxel and starved cells and this association was increased in the presence of bafilomycin.

Figure 2. Mitotically arrested cells show diminished autophagosome formation. MCF-7 cells stably expressing GFP-LC3 were plated on a 35 mm glass bottom culture dish and treated with 25 nM paclitaxel. Image stacks were acquired once per hour. Shown are two fields of view acquired from two independent experiments (upper and lower). Arrows indicate a cell entering into mitotic arrest, and arrowheads denote the resulting daughter cells. After deconvolution, the amount of fluorescence localized within the puncta was quantified and presented above each image as a percentage of the total cellular GFP-LC3 fluorescence. The experiment was performed independently three times and representative images are shown.
However, in the presence of paclitaxel, there was diminished association of autophagosomes with lysosomes. This diminished association was highlighted by comparing fluorescent intensity peaks from GFP-LC3 and LAMP1 staining (Figure 6, intensity histograms, black arrowheads). Thus, paclitaxel-mediated aberrations in autophagosome movement and trafficking inhibited autophagosome fusion with lysosomes.

**Figure 3.** Mitotically arrested cells have decreased autophagic flux. (a) MCF-7 cells stably expressing GFP-LC3 were treated with 25 nM paclitaxel for 24 h. After treatment the plate was knocked repeatedly to dislodge loosely attached cells and those cells were collected onto a microscope slide, stained with 4,6-diamidino-2-phenylindole (DAPI), and imaged. The experiment was performed independently twice, and representative images are shown. (b) MCF-7 and SK-BR-3 cells were left untreated or treated with 25 nM paclitaxel for 24 h. For the last 4 h of paclitaxel treatment, one plate of each duplicate was treated with 100 nM bafilomycin A1. At the time of harvest, the floating (mitotically arrested) cells in the paclitaxel-treated samples were collected and lysed separately from the adherent cells. Protein content was quantified, and 20 μg of each lysate was loaded onto a sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and separated by electrophoresis. Western blots were then performed for the indicated proteins. The experiment was performed independently three times and representative blots are shown.

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**Autophagy contributes to paclitaxel cytotoxicity**

Given that autophagy has controversial roles in cell survival and cell death, we tested whether paclitaxel-mediated inhibition of autophagy affected paclitaxel cytotoxicity. We treated cells with paclitaxel in the presence and absence of the Vps34 inhibitor 3-methyladenine (3MA) and the level of apoptosis was determined by staining with the mitochondrial potentiometric dye, tetramethyl rhodamine ethyl ester (Figure 7a). Paclitaxel treatment resulted in a specific cell death of 25%, which was calculated as amount of cell death of paclitaxel-treated cells minus the amount of spontaneous cell death in control untreated cells. Inhibiting autophagosome formation significantly reduced this paclitaxel-induced cell death from 25 to 14%. 3MA treatment alone can induce apoptosis, thus when this 3MA-dependent cytotoxic effect was taken into account, cell death specific to paclitaxel was...
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Figure 4. The Vps34 complex is inhibited in mitotically arrested cells. MCF-7 and SK-BR-3 cells were left untreated, treated with 25 nM paclitaxel for 24 h, or starved for 4 h. At the time of harvest, the floating (mitotically arrested) cells in the paclitaxel-treated samples were collected and lysed separately from the adherent cells. Protein content was quantified, and 30 μg of each lysate was loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and separated by electrophoresis. Western blots were then performed for the indicated proteins. The experiment was performed independently three times and representative blots are shown.

Reduced to 8%. We repeated the same experiments in the SK-BR-3 cell line. Paclitaxel triggered 67% specific cell death of SK-BR-3 cells, which was significantly reduced to 47% in the presence of 3MA. When the 3MA-dependent cytotoxic effect was taken into account, cell death specific to paclitaxel was further reduced to 41%. We also evaluated paclitaxel-induced cell death with a clonogenic assay (Figure 7b). 3MA treatment of MCF-7 cells resulted in an 8.3-fold increase in clonogenic survival of paclitaxel-treated cells. Similarly, 3MA increased the clonogenic survival of SK-BR-3 cells by 6.1-fold. To confirm these findings, we used small interfering RNA (siRNA) against the autophagy gene products of Atg7 and Vps34 and tested paclitaxel sensitivity (Figure 7c). MCF-7 cells transfected with siRNA against Atg7 showed reduced paclitaxel-induced cell death to 76% of cells transfected with nonspecific siRNA. As well, SK-BR-3 cells transfected with siRNA against Vps34 showed 77% sensitivity to paclitaxel treatment relative to control cells. Thus, inhibition of autophagosome formation reduced paclitaxel-induced cell death.

A subset of autophagy markers is downregulated in docetaxel-resistant breast tumors
Based on the previous data suggesting that autophagy contributed to paclitaxel-induced apoptosis, we postulated that autophagy gene expression could be downregulated in taxane-resistant breast tumors. In order to address this question, we queried a data set from a previous study conducted by Chang et al.26 We showed that paclitaxel inhibited both autophagosome formation in mitotic cells, and autophagosome trafficking (see Figure 8, model). When we used 3MA and siRNAs against ATG7 and VPS34 to block autophagosome formation, paclitaxel-induced apoptosis was diminished, indicating that autophagy contributes to paclitaxel cytotoxic effects. This autophagy-dependent death may be due to activation of autophagy-related cell death (PCDII). However, this seems unlikely, as we showed that paclitaxel blocked autophagic flux, which would block PCDII. Rather, our data support a model whereby blockage of autophagosome trafficking and maturation contributes, through an unknown way, to cell death. This may be of clinical importance, since we observed that a subset of genes required for autophagosome formation was downregulated in taxane-resistant primary breast tumors.

DISCUSSION
We showed that paclitaxel inhibited both autophagosome formation in mitotic cells, and autophagosome trafficking (see Figure 8, model). When we used 3MA and siRNAs against ATG7 and VPS34 to block autophagosome formation, paclitaxel-induced apoptosis was diminished, indicating that autophagy contributes to paclitaxel cytotoxic effects. This autophagy-dependent death may be due to activation of autophagy-related cell death (PCDII). However, this seems unlikely, as we showed that paclitaxel blocked autophagic flux, which would block PCDII. Rather, our data support a model whereby blockage of autophagosome trafficking and maturation contributes, through an unknown way, to cell death. This may be of clinical importance, since we observed that a subset of genes required for autophagosome formation was downregulated in taxane-resistant primary breast tumors.

These results suggested that these autophagy markers may be useful prognostic indicators for chemotherapeutic taxane responsiveness.

Paclitaxel inhibits autophagy
We showed that paclitaxel inhibits autophagy in MCF-7 and SK-BR-3 breast carcinoma cell lines. These data are in conflict with other reports wherein a paclitaxel-mediated increase in autophagosome numbers was interpreted as stimulated autophagy.5,18 However, because of autophagosome turnover, an increase in autophagosome numbers is not on its own, indicative of an increase in autophagic flux. By inhibiting lysosomal acid hydrolases with bafilomycin, we determined that autophagosomes did not mature to autophagolysosomes indicating that autophagy was in fact blocked. Furthermore, by visualizing autophagosome formation and movement over time, we directly observed that while autophagosomes formed in non-mitotic cells, autophagic flux was inhibited.

To put our results in context with previous conflicting reports examining the effects of mitotic/microtubule inhibitors on autophagy, we suggest that mitotic-inhibiting agents are general inhibitors of autophagy. Early studies using vinblastine interpreted increased numbers of autophagosomes as evidence of increased levels of autophagy.19,20 However, these studies lacked the lysosomal inhibitor controls needed to differentiate increased autophagosome production from decreased autophagosome degradation. Furthermore, electron microscope examination of autophagosome structures after vinblastine treatment showed an absence of degraded organelles until 12 h after treatment. As comparable experimental systems demonstrated organelle half-life of <30 min,21,22 this suggests that instead, vinblastine inhibits autophagosome maturation. In support of this, more recent studies using lysosomal inhibitor controls showed that nocodazole, vinblastine and paclitaxel inhibited autophagosome formation or maturation.23-25 Our work extends these studies to use of clinically relevant low-dose treatment of paclitaxel and demonstrate an inhibition of autophagy.

Inhibition of autophagy in mitotic cells is also a controversial issue. Liu et al.26 observed robust autophagy in mitotic HeLa cells, whereas Eskelinen et al.27 and Furuya et al.16 observed suppressed autophagy in normal mitotic and nocodazole-enriched mitotic NRK, H4, HeLa and 293T cells. We found that treatment of breast carcinoma cells with paclitaxel, inhibited autophagy in mitotic cells. This was demonstrated through biochemical analysis of LC3-II levels and live cell microscopy of GFP-LC3-labeled autophagosomes. Thus, we suggest that paclitaxel-induced inhibition of autophagy is dependent on mitosis. Moreover, we demonstrated that inhibition of autophagy was a
result of inhibited autophagosome formation mediated in part through inhibition of the critical kinase Vps34.

Paclitaxel inhibits autophagosome trafficking
Paclitaxel causes mitotic arrest followed by aberrant mitotic exit.\(^{14,15,28,29}\) Interestingly, this mitotic slippage is associated with apoptosis in MCF-7 cells.\(^{14}\) We showed that as paclitaxel-treated cells escaped mitotic arrest, there was a re-activation of autophagosome formation, however, autophagosome maturation was blunted. This was due in part to a defect in autophagosome trafficking. Previous groups demonstrated that disruption of microtubule structure inhibits autophagosome trafficking.\(^{23,25,30,31}\) However, the findings with the microtubule-stabilizing agent, paclitaxel, are less clear and are confounded by the fact that paclitaxel concentration mediates different cellular responses. Low-dose paclitaxel concentrations (5–200 nM), which were used in our study, are defined as clinically relevant\(^{32}\) based on the determination of the concentration of intracellular drug accumulation\(^{33}\) and clinically achievable plasma levels.\(^{34}\) Within this range, paclitaxel-treated cells induce an apoptotic program that is dependent on mitotic arrest.\(^{35}\) On the other hand, cells treated with high concentrations of paclitaxel (3–100 \(\mu M\)) show increased microtubular polymerization and massive microtubule damage\(^6\) and undergo both apoptosis and necrosis in a cell cycle stage-independent fashion.\(^{35}\) With respect to high-dose paclitaxel treatment, Kochl et al.\(^{25}\) found that paclitaxel had a negligible effect on autophagosome trafficking in hepatocytes, whereas Kimura et al.\(^{36}\) found decreased autophagosome movement in HeLa cells. These conflicting results may reflect cell-type-specific differences. From our studies with clinically relevant low-dose paclitaxel treatment, we conclude that paclitaxel-treated breast cancer cells had a block in autophagosome trafficking. Importantly, by using longer time points that take into account the secondary effects encompassing cell cycle-influenced effects, we were able to discern two distinct blocks in the autophagy pathway.

Autophagy as a cell death mechanism
We identified that paclitaxel transiently inhibited autophagosome formation. Paclitaxel lessened autophagosome formation in mitotic cells, while cells that slipped out of mitotic arrest regained the ability to produce autophagosomes that were not properly moved. This was due in part to a defect in autophagosome trafficking. Previous groups demonstrated that disruption of microtubule structure inhibits autophagosome trafficking.\(^{23,25,30,31}\) However, the findings with the microtubule-stabilizing agent, paclitaxel, are less clear and are confounded by the fact that paclitaxel concentration mediates different cellular responses. Low-dose paclitaxel concentrations (5–200 nM), which were used in our study, are defined as clinically relevant\(^{32}\) based on the determination of the concentration of intracellular drug accumulation\(^{33}\) and clinically achievable plasma levels.\(^{34}\) Within this range, paclitaxel-treated cells induce an apoptotic program that is dependent on mitotic arrest.\(^{35}\) On the other hand, cells treated with high concentrations of paclitaxel (3–100 \(\mu M\)) show increased microtubular polymerization and massive microtubule damage\(^6\) and undergo both apoptosis and necrosis in a cell cycle stage-independent fashion.\(^{35}\) With respect to high-dose paclitaxel treatment, Kochl et al.\(^{25}\) found that paclitaxel had a negligible effect on autophagosome trafficking in hepatocytes, whereas Kimura et al.\(^{36}\) found decreased autophagosome movement in HeLa cells. These conflicting results may reflect cell-type-specific differences. From our studies with clinically relevant low-dose paclitaxel treatment, we conclude that paclitaxel-treated breast cancer cells had a block in autophagosome trafficking. Importantly, by using longer time points that take into account the secondary effects encompassing cell cycle-influenced effects, we were able to discern two distinct blocks in the autophagy pathway.

Figure 5. Paclitaxel treatment inhibits autophagosome movement. (a) MCF-7 cells stably expressing GFP-LC3 were left untreated or treated with 25 nM paclitaxel for 24 h. The cells were then examined on a spinning disk confocal microscope with 2 stacks acquired at maximum speed (approximately 10 s per stack). The initial position of the GFP-LC3 puncta are shown (top) as well as their paths over the subsequent 5 min (bottom). (b) The average speed of each puncta was determined and the percentage of puncta for each range of speeds was plotted. The experiment was repeated independently three times and a total of 1200 puncta (in non-mitotic cells) were tracked. Error bars indicate the s.d. (c) The overall puncta speed from the three independent experiments shown in (b) were represented as an average value, relative to untreated cells. Error bars indicate the s.d.
trafficked. When we further blocked autophagosome formation with 3MA or siRNA against ATG7 or VPS34, paclitaxel-induced apoptosis was diminished. These results indicate that autophagy contributed to taxane-induced cell death, although the mechanism is unclear. It is unlikely that excessive autophagy (PCDII) is involved, as we showed that autophagosome maturation is blocked by paclitaxel, and the idea that PCDII is an independent means of cell death is currently a topic of debate. Instead, we propose that inhibition of autophagosome trafficking/turnover may itself be a novel trigger of cell death. Therefore, in the presence of paclitaxel, autophagosome formation after mitotic slippage results in a buildup of autophagosomes that cannot be degraded. The subsequent arrest in autophagosome maturation thus links blocked autophagy to apoptosis.

Finally, our data support a connection between autophagy and clinical drug effectiveness, whereby blunted autophagy is associated with positive response to taxane therapy. Autophagy genes/proteins could be investigated as possible prognostic, or predictive markers for clinical taxane effectiveness. As taxanes are widely used chemotherapy agents, it now becomes critically important to understand the interplay between taxane treatment, autophagy and cell death.

**MATERIALS AND METHODS**

**Chemicals and antibodies**

All chemicals were obtained from the Fisher Scientific Company (Nepean, ON, Canada) unless otherwise noted. 3MA, bafilomycin A1 and paclitaxel were obtained from Sigma-Aldrich (Ottawa, ON, Canada). Tetramethyl rhodamine ethyl ester and Hoechst 33342 were obtained from Invitrogen (Carlsbad, CA, USA). FuGENE 6, and protease/phosphatase inhibitors were obtained from Roche (Indianapolis, IN, USA). Validated siRNA oligonucleotides against ATG7 and VPS34 were from Qiagen (Valencia, CA, USA) and transfected into cells using Interferin (Polyplus, New York, NY, USA) according to the manufacturer’s instructions. Alexa Fluor fluorescent secondary antibodies were purchased from Invitrogen (A31571, A31572). Antibodies LC3-B and tubulin were purchased from Sigma-Aldrich (L7543, T5168). Vps34 (T159) antibody was a kind gift from Dr Junying Yuan (Harvard University, Cambridge, MA, USA). GFP antibody (EU1) was a kind gift from Dr Luc Berthiaume (Eusera, University of
Figure 7. Autophagy contributes to paclitaxel-induced cell death. (a) MCF-7 and SK-BR-3 cells were left untreated or treated with 25 nM paclitaxel and/or 5 mM 3MA for 48 h. Cells were harvested, stained with tetramethyl rhodamine ethyl ester (TMRE), and then analyzed by flow cytometry. Spontaneous background cell death is subtracted from all values. The mean of three independent experiments is plotted as a bar graph with error bars denoting the standard error. P-values were calculated with using the Student's two-tailed t-test. (b) MCF-7 and SK-BR-3 cells were treated similar to A, after which 2000 cells from each treatment were plated and incubated for 3 weeks before fixation and staining with crystal violet. The experiment was performed three independent times and a representative assay is shown. The average number of colonies for all three independent experiments is shown as a bar graph with error bars denoting the s.d. P-values were calculated using the Student's t-test. (c) siRNA oligonucleotides against ATG7, VPS34 and nonspecific (NS) sequence were transfected into MCF-7 and SK-BR-3 cells as shown. Cells were left untreated or treated with 25 nM paclitaxel for 48 h and TMRE fluorescence was analyzed by flow cytometry. Spontaneous background cell death was subtracted from cell values. The cell death relative to control untreated samples of three independent experiments was plotted as a bar graph with error bars denoting the s.d. P-values were calculated using the Student's t-test. (d) In all, 24 primary breast tumor biopsy samples were divided into docetaxel-resistant or docetaxel-sensitive categories, and genome wide miRNA microarray data was generated for each tumor. The microarray data were queried for expression levels of autophagy-related genes. Gene names are indicated on the left. The Atg12 microarray values are an average change in expression of three independent primers for the same gene. P-values from Student’s t-test are found to the right on the right. Microarray data from Chang et al.17
Figure 8. Paclitaxel inhibits autophagy at two stages. Paclitaxel-induced mitotic arrest causes decreased autophagic flux through phosphorylation and inhibition of Vps34. Additionally, paclitaxel causes aberrant autophagosome trafficking and localization, inhibiting degradation of autophagosomes.

Alberta, Edmonton, AB, Canada). LAMP1 antibody (SC-20011) was purchased from Santa Cruz (Santa Cruz, CA, USA). P62 antibody (610832) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Pericentrin antibody was a kind gift from Dr Gordon Chan (University of Alberta). Horseradish peroxidase - conjugated secondary antibodies (170-6515, 170-6516) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Pericentrin antibody was a kind gift from Dr Gordon Chan (University of Alberta). Horseradish peroxidase - conjugated secondary antibodies (170-6515, 170-6516) were purchased from Bio-Rad (Mississauga, ON, Canada).

Cell culture
Human breast cancer cell lines (MCF-7 and SK-BR-3) were obtained from Dr Gordon Mills (MD Anderson Cancer Center, University of Texas, Houston, TX, USA). MCF-7 cells stably expressing GFP-LC3 were created by transfecting parental cells with pEGFP-C1 vector containing rat LC3-B complementary DNA (a gift from Dr Gordon Shore, McGill University, Montreal, QC, Canada) followed by clonal selection.

Apoptosis assays
Breast cancer cells were treated with 25 nM paclitaxel or left untreated, then harvested, stained with tetramethyl rhodamine ethyl ester, and analyzed by flow cytometry as described previously. (39)

Microarray analysis
Microarray data generated from docetaxel-sensitive or -resistant tumors (GEO data set GDS360) were obtained from the NCBI GEO database. (17, 40) Raw data were imported into Excel (Microsoft, Redmond, WA, USA), then normalized, and changes in expression were expressed as log2 values, which were then color coded and presented as a heat map.

Live cell microscopy
For wide field live cell microscopy, cells were plated on 35 mm glass bottom culture dishes (MatTek, Ashland, MA, USA) and imaged on a Zeiss Axio Observer Z1 (Carl Zeiss Canada, Toronto, ON, Canada) equipped with a PeCon Heating Insert P S with PM CO2 cover (PeCon GmbH, Erbach, Germany). For confocal live cell microscopy, cells were plated in a Lab-Tek II Chambered Coverglass (Nunc, Rochester, NY, USA) and imaged on an UltraView VoX Confocal Imaging System (PerkinElmer, Woodbridge, ON, Canada) equipped with a Universal ASI Stage Water Jacketed Incubator and CO2 atmosphere system (Okolab, NA, Italy). Excitation radiation was provided by a 488 nm laser and emitted radiation was collected through a 527 nm (50 nm) band pass filter (PerkinElmer).

Deconvolution and microscopic data analysis
After acquisition, widefield stacks were deconvolved using Huygens Professional (Scientific Volume Imaging, Hilversum, The Netherlands) and visualized in Imaris × 64 (Bitplane Scientific Software, Zurich, Switzerland). Puncta counting was performed blindly by using randomized filenames (Filename randomizer, CodeUnit, Craig Lotter) and puncta were counted by generating surfaces in Imaris, then counting the resulting surfaces. To determine the percentage of fluorescence localized to puncta, a surface was generated over the entire cell, followed by surfaces for all of the puncta. The percentage was determined by dividing the total fluorescence found in puncta by the total fluorescence within the cell.

Statistical analysis
Data are presented as a mean of three independent experiments, with error bars indicating the s.d. Statistical significance was determined using a two-tailed Student's t-test for two means with equal variance.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES


