Carcinogen treatment leads to mitotic defects and arrest in cancer and noncancer cells

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Genomic instability can manifest due to both chromosomal rearrangements and gain and loss of entire chromosomes. One mechanism by which a carcinogen acts is by increasing the rate of mitotic spindle defects during proliferation. These defects can lead to chromosomal instability that manifest as lagging chromosomes, anaphase bridges, or multipolar spindles. While several mechanisms exist to rectify these errors prior to completion of mitosis, some cells will escape repair, while others will prematurely exit mitosis. Here we examine the effects of two carcinogenic molecules: Fulvestrant, a chemotherapeutic that functions as a selective estrogen receptor degrader, and vinyl chloride, a hydrocarbon used to produce PVC. We exposed two cancer lines, A549 and UPCI:SCC103, and one noncancer line, GM03349, to increasing concentrations of the carcinogen for increasing durations, up to 48 hours exposure. We found that exposure to the carcinogen lowered the mitotic index in the cancer cell lines, while raising it in the noncancer line. Concurrently, we observed massive increases in the frequency of mitotic defects, with the most significant increases seen in prevalence of lagging chromosomes in prometaphase and metaphase and anaphase bridges. Live cell imaging showed that the occurrence of either of these defects had the strongest correlation with the likelihood that the cell would fail to complete mitosis. We also show that washing out the carcinogen decreases the frequency of mitotic defects in all three cell lines, but the mitotic index does not recover in the cancer cells. These findings demonstrate that carcinogen-induced mitotic defects have marked effects on the proliferative population of cells in terms of potential for contributing to chromosomal instability or removal from that population.

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Abstract

Genomic instability can manifest due to both chromosomal rearrangements and gain and loss of entire chromosomes. One mechanism by which a carcinogen acts is byincreasing the rate of mitotic spindle defects during proliferation. These defects can lead to chromosomal instability that manifest as lagging chromosomes, anaphase bridges, or multipolar spindles. While several mechanisms exist to rectify these errors prior to completion of mitosis, some cells will escape repair, while others will prematurely exit mitosis. Here we examine the effects of two carcinogenic molecules: Fulvestrant, a chemotherapeutic that functions as a selective estrogen receptor degrader, and vinyl chloride, a hydrocarbon used to produce PVC. We exposed two cancer lines, A549 and UPCI:SCC103, and one noncancer line, GM03349, to increasing concentrations of the carcinogen for increasing durations, up to 48 hours exposure. We found that exposure to the carcinogen lowered the mitotic index in the cancer cell lines, while raising it in the noncancer line. Concurrently, we observed massive increases in the frequency of mitotic defects, with the most significant increases seen in prevalence of lagging chromosomes in prometaphase and metaphase and anaphase bridges. Live cell imaging showed that the occurrence of either of these defects had the strongest correlation with the likelihood that the cell would fail to complete mitosis. We also show that washing out the carcinogen decreases the frequency of mitotic defects in all three cell lines, but the mitotic index does not recover in the cancer cells. These findings demonstrate that carcinogen-induced mitotic defects have marked effects on the proliferative population of cells in terms of potential for contributing to chromosomal instability or removal from that population.

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Introduction

Genomic instability is defined as a change in chromosome structure or number and is observed in many developing tumors. One of the most prevalent ways in which genomic instability manifests is via chromosomal instability (CIN). This often arises via mis-segregation of chromosomes during mitosis resulting in the gain or loss of genetic material by the daughter cells. In addition to CIN, which refers to whole chromosome rearrangements, other defects can be observed in the chromosome, such as deletions, amplifications, inversions and translocations. The causes of these genomic perturbations are broad and likely stem from changes in DNA structure, regulation of the cell cycle or other cellular metabolic changes (Balanis & Graeber 2018; Elledge 1996; Miyagawa 1998; Nambiar et al. 2008; Winey 1996). These genetic errors can lead to aneuploidy and progression of tumor cell stage (Balanis & Graeber 2018; Gao et al. 2007; Lengauer et al. 1998; Levine & Holland 2018).

Aneuploidy has long been tied to mitotic defects in tumor cells: in the late 19th and early 20th centuries, both von Hansemann and Boveri observed defects in chromatin during mitosis (Maderspacher 2008; Wunderlich 2011). There are three main types of mitotic defects that are commonly observed. First, multipolar spindles are characterized by the appearance of more than two spindle poles that pull the chromatin in multiple directions due to the presence of extra centrosomes (Ghadimi et al. 2000; Lingle et al. 2002). A cell may acquire extra centrosomes by several means including extra rounds of duplication or failure of cytokinesis during a previous division (Ganem et al. 2005). While the cell possesses a mechanism to prevent multipolarity, many cancer cells may evade this defense system (Kwon et al. 2008; Quintyne et al. 2005). Second, anaphase bridges can form when non-telomeric ends of chromosomes are fused together

resulting in a super-chromosome, containing more than one centromere. These structures may sometimes break during anaphase, forming a second set of broken DNA ends, which will be fused once again undergoing the breakage-fusion-bridge cycle (BFB) (McClintock 1941). A more recent study, though, indicates that many bridges will not break and will instead persist into the next cell cycle, still contributing to aneuploidy (Pampalona et al. 2016). Third, lagging chromosomes are either full or fragmented chromosomes set apart from the main chromatin mass during metaphase and anaphase. These often end up as micronuclei upon the completion of mitosis. The composition of the lagging chromosome may be derived from a chromosome that failed to attach properly to the spindle, an acentric chromosome fragment that is produced by a double-stranded break prior to mitosis, or from two breaks along the length of an anaphase bridge (Luo et al. 2004; Saunders et al. 2000). Misregulation of many proteins has been implicated in the formation of lagging chromosomes, including APC (Fodde et al. 2001), pRB (Manning et al. 2010), BRCA1 (Joukov et al. 2006; Stolz et al. 2010), Bub1 (Ricke et al. 2011) CDC4 (Rajagopalan et al. 2004), or STAG4 (Kleyman et al. 2014). A recent analysis of breast tumor genomes has demonstrated that overexpression of the transcriptional regulators MYBL2, E2F1, and FOXM1 increases the rate of lagging chromosomes and aneuploidy (Pfister et al. 2018). Meanwhile knock down of the DNA damage repair protein ATR or mutations in its sequence will lead to an increase in the rate of lagging chromosomes (Kabeche et al. 2018).

The mechanism by which each of these defects contribute to chromosomal instability and thus aneuploidy and cancer progression is a complex picture involving misregulation of a variety of proteins. While anaphase bridges cause rearrangements of parts of the chromosome, multipolar spindles would appear to be a source of large-scale changes to the genome. However, such changes are likely sufficient to kill any progeny cell, and thus the contribution of a

successfully dividing multipolar cell is questionable. Previous research has shown that few multipolar cells will complete mitosis, and a multipolar cell that does successfully divide produces cells that are usually nonviable. The population of those cells that are viable rarely divide more than one additional time (Ganem et al. 2009). Furthermore, it was demonstrated that multipolar spindles contain numerous merotelic attachments, and that multipolarity represents an intermediate state associated with high levels of merotelic connections (Ganem et al. 2009).

These merotelic connections are poorly recognized by the spindle assembly checkpoint, and can lead to lagging chromosome formation (Cimini 2008; Cimini et al. 2001; Cimini et al. 2003; Salmon et al. 2005; Thompson et al. 2010). In any cellular population exhibiting mitotic defects, lagging chromosomes tend to be the most commonly observed phenomena, usually because of these merotelic attachments (Thompson & Compton 2008). In addition to the presence of extra centrosomes, induction of lagging chromosomes can occur via several other mechanisms, including DNA breaks, formation of DNA adducts and cellular cytotoxicity (Armstrong et al. 2000; Ding et al. 2003; Holz et al. 1995; Stewenius et al. 2005).

These mitotic defect-inducing cellular changes can be produced by a variety of carcinogens. The subsequent mitotic defect is often dependent on how the carcinogen interacts with the cell, and more specifically DNA. Some carcinogens can produce reactive oxygen species (ROS) that will directly interact with DNA, causing breaks in the sugar-phosphate backbone (Pelicano et al. 2004; Toyokuni et al. 1995). Two single-stranded breaks in close proximity will lead to a double-stranded break, which can lead to anaphase bridges or acentric lagging chromosomes (Luo et al. 2004). Additionally, ROS can function as signaling molecules in proliferative pathways (Pelicano et al. 2004). Other carcinogens interact with the nitrogenous base of the DNA, forming a DNA adduct. This mis-shaping of DNA can have consequences for

replication, repair and normal chromosomal function. Additionally, it has been observed that DNA adducts can recruit subunits of topoisomerase, which can lead to DNA cleavage (Velez-Cruz et al. 2005). Furthermore, carcinogens can lead to cytotoxicity, which may increase oxidative stress on the cells, and can lead to an increase of ROS as well as other cellular effects (Armstrong et al. 2000; Holz et al. 1995). Carcinogens can also function by inhibiting important cell cycle regulatory machinery, such as p53 (Thompson & Compton 2010).

Here, we wanted to determine if direct exposure to carcinogen would phenocopy the mitotic defects observed as a result of induced changes of gene expression or induced tetraploid cells with extra centrosomes, i.e. an increase in lagging chromosomes. To examine this, we used two different carcinogens: First, we used vinyl chloride, a chlorinated aliphatic hydrocarbon used during the industrial production of polyvinyl chloride (PVC), which has been linked to a number of different cancers (Maltoni et al. 1981; Viola et al. 1971). Vinyl chloride forms a DNA adduct and has been shown to increase topoisomerase I activity (Pourquier et al. 1998; Velez-Cruz et al. 2005). Second, we used Fulvestrant (Faslodex), a medication used in the treatment of hormone receptor positive metastatic breast cancer (Battista & Schmidt 2016). Fulvestrant is a selective estrogen receptor degrader (SERD) that works by binding to the estrogen receptor on the surface of a tumor cell and destabilizing it. This destabilization occurs because Fulvestrant will make the receptor more hydrophobic, leading to misfolding of the receptor, thus resulting in a loss of estrogen signaling and a decrease in proliferative rate (Howell et al. 2004). However, when estrogen receptors are degraded, p53 is downregulated, leaving the genome and the cell susceptible to damage (Berger et al. 2012). Previous research has shown that p53 activity is necessary to limit rates of chromosomal instability and aneuploidy (Thompson & Compton 2010).

We tested our two carcinogens in three different cell lines: two derived from tumor tissue and one that is noncancerous. First, we used the adenocarcinomic human alveolar basal epithelial cells, A549, a hypotriploid cell line that displays mitotic defects in culture (Giard et al. 1973; Loffler et al. 2013; Park et al. 2017; Quintyne et al. 2005). Second, we used the human oral squamous carcinoma cell line UPCI:SCC103, which has been demonstrated to be aneuploid and also exhibits high levels of mitotic defects (Quintyne et al. 2005; Reing et al. 2004; Saunders et al. 2000). Third, we used GM03349, a genomically normal skin fibroblast cell line that exhibit low levels of mitotic defects (Budel & Djabali 2017; Luo et al. 2004; Scudiero et al. 1981). We observed that brief exposure to carcinogen increased the mitotic index of GM03349 cells, but decreased the mitotic index in the two cancer lines. Additionally, we saw an increase in the rates of mitotic defects in all cell lines particularly lagging chromosomes in prometaphase/metaphase and anaphase bridges. Furthermore, cells treated with vinyl chloride that exhibited lagging chromosomes generally failed to complete mitosis, and cells with anaphase bridges also failed to complete mitosis nearly half the time (44% in A549 and UPCI:SCC103, 50% in GM03349). We also observed that when we washed out the carcinogen from our samples, defect frequencies decreased to nearly pre-exposure levels, but the mitotic index remained low in our cancer cell lines. Altogether, we demonstrate that brief exposure to carcinogens is sufficient to trigger mitotic defects that have effects on the replicative state of the cancer cells beyond the initial exposure.

Methods

Cell Culture

The adenocarcinomic human alveolar basal epithelial cells, A549, were obtained from the American Type Culture Collection (ATCC©, Manassas VA). These cells were cultured using Ham's F-12K Kaighn's Medium (Gibco, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (Corning Life Sciences, Lowell, CA), and Penicillin-Streptomycin (Corning). The oral squamous carcinoma cell line UPCI:SCC103 were a gift from Susan Gollin (University of Pittsburgh Cancer Institute), and were cultured using Minimal Essential Medium (Gibco) supplemented with 10% Fetal Bovine Serum, Non-Essential Amino Acids(Gibco) L-Glutamine (Gibco), and Gentamycin (Gibco). The normalized non-fetal skin fibroblast line, GM03349, was obtained from Coriell Cell Repositories (Camden, NJ). This line was cultured in Dulbecco's Modified Eagle Medium (Gibco) that was supplemented with 10% Fetal Bovine Serum and Penicillin-Streptomycin. All cells were grown at at 37°C in a 5% CO_2 humidified environment. Cells were passaged using trypsin-EDTA (0.25% trypsin for A549 and GM03349, 0.05% trypsin for UPCI:SCC103) when the cells were no greater than 85% confluent.

Chromatin Staining

Coverslips were fixed with 1 ml of room temperature 4% paraformaldehyde for 30 minutes. The paraformaldehyde was removed and the coverslips washed with phosphate buffered saline (PBS) for five minutes three times. Then 150 µl of 4',6-diamidino-2-pheylindole (DAPI; Invitrogen, Carlsbad, CA) was added and incubated for 30 seconds. The coverslips were then washed 3

161 times with milliQ water. Coverslips were then mounted onto glass slides with a drop of mounting
162 media (p-phenyldiamine, PBS, Glycerol).
163
164 Carcinogen Treatments
165 A stock solution of Fulvestrant was prepared by adding 0.006 grams of powdered
166 fulvestrant (Sigma-Aldrich, St Louis, MO) into 10 ml of Dimethyl Sulfoxide (DMSO; Sigma-
167 Aldrich, St Louis,MO). The fulvestrant-DMSO solution was then diluted into the appropriate
168 growth medium for each cell line to produce dilutions of 10 µM, 20 µM, 50 µM, and 100 µM.
169 156.2 µl of 2 mg/ml Vinyl Chloride (Restek, CAT#30089, Bellefonte, PA) was diluted into 50
170 ml of cell culture media to make a 100 µM stock solution, which was then diluted in the
171 appropriate growth medium for each cell line to generate 5 µM, 10 µM, 20 µM, and 50 µM
172 solutions. Coverslips were seeded for each cell line and allowed to grow normally for 24 hours.
173 The media was then removed and replaced with a specific concentration of carcinogen-
174 containing media. This media was left on for 24, 36, or 48 hours before coverslips were fixed
175 and slides prepared.
176
177 Cell Counting
178 To visualize the coverslips either a Leica DM 2000 Fluorescent microscope (100x oil objective,
179 N. A. = 1.25; Wetzlar, Germany) or a Leica SP8 Laser Scanning Confocal microscope (63x oil
180 objective, N.A. = 1.40) was utilized using the correct filter settings for DAPI staining. For
181 mitotic index counts, at least 500 cells were counted per coverslip and the percentage of mitotics
182 recorded. For defect indices, at least 50 mitotic cells were scored per slide and the percentages of
183 cells exhibiting each class of mitotic defect were recorded. For micronucleus indices, at least 500

interphase cells were counted per coverslip for each condition/cell line. Statistical analysis using a student t-test was performed between the untreated control and each experimental condition independently to determine if there was a statistically significant difference between treated and untreated cells.

Live Cell Analysis

Cells were seeded on a glass bottom 35 mm^2 tissue culture plate (MatTek CAT#P35G-0.170-14-C, Boston MA) and grown either as controls or with carcinogen treatments as previously described. Cells were stained by placing 1 µL of SiR-DNA (SiR-Hoechst; Cytoskeleton, Denver CO) into the cellular media and incubating for 45 minutes at 37°C. The plate was transferred to a PeCon 2000 stage top incubator (PeCon, Erbach, Germany) mounted on the Leica SP8 Laser Confocal Microscope. The chamber was set to 37°C with 5% CO_2 and humidity. Cells were then observed with a 63x water immersion objective (N.A.=1.20) using far-red laser/detector settings as described by the SiR-DNA manufacturer. Live cell movies were generated using Leica Application Suite advanced Fluorescence software version 4.0 by taking sets of Z-stack images every three minutes for at least eight hours. 3D renderings were created from the Z-stack time courses for analysis. Mitotic cells were analyzed frame-by-frame to determine if defects were present.

Cell Viability

In order to determine the percentage of live cells in each population a viability assay was performed. The collected pellet of cells was resuspended in 200µL of PBS then briefly vortexed and 20µL was transferred to a new tube. Next, 380µL of Guava ViaCount© reagent by EMD

Millipore (Billerica MA) was added and vortexed briefly. The suspension was incubated for 5 minutes in the dark and again vortexed briefly. The sample was then loaded into the Guava easyCyte flow cytometer (Millipore, Billerica MA) and analyzed using ViaCount software, with 1000 events (cells) counted for each sample.

Results

Exposure to Fulvestrant or Vinyl Chloride decreases viability of noncancer cells at concentrations of 100 μM

To examine the effects of short-term carcinogen exposure on mitotic progression, we chose to use two carcinogens that affect cells in different ways. First, we chose Fulvestrant, a selective estrogen receptor degrader whose activity will downregulate p53 activity within the cell via destabilization of the estrogen receptor (Battista & Schmidt 2016; Berger et al. 2012); p53 activity has been demonstrated to be necessary for limitation of chromosomal instability and aneuploidy (Thompson & Compton 2010). Second, we chose to use vinyl chloride, an industrial byproduct of PVC production that generates DNA adducts and increases topoisomerase I activity (Pourquier et al. 1998; Velez-Cruz et al. 2005). We selected three cell lines to expose to our two carcinogens, two cancer lines and a noncancer line. First, we used A549 cells, an adenocarcinomic human aveolar basal epithelial line, which is hypotriploid and has a high level of mitotic defects present (Giard et al. 1973; Loffler et al. 2013; Park et al. 2017; Quintyne et al. 2005). Second, we used the oral squamous cell carcinoma line UPCI:SCC103, an aneuploid line that has been shown to not only display high levels of mitotic defect in culture, but also exhibits

a consistent level of mitotic defect from generation to generation (Reing et al. 2004). For our noncancer cell line, we used GM03349 normal skin fibroblasts that do not show high levels of mitotic defects (Budel & Djabali 2017; Luo et al. 2004; Scudiero et al. 1981).

We exposed each cell line to four different concentrations of each carcinogen (10 μM, 20 μM, 50 μM, and 100μM) for 24, 36, and 48 hours before collecting all cells and determining the viability of each cell line and condition using ViaCount reagent and software on a Guava easyCyte flow cytometer. Overall, we observed no change in viability at any concentration or time in our two cancer cell lines with either Fulvestrant (Figure 1A-C) or vinyl chloride (Figure 1D-F). However, we saw a moderate decrease in viability of GM03349 cells treated with 50 μM Fulvestrant for 48 hours (Figure 1C) and cells treated with 50 μM vinyl chloride for 36 or 48 hours (Figure 1E-F). However, we observed a large (30-40%) decrease in viability of GM03349 cells at 100 μM concentrations of both Fulvestrant and vinyl chloride at all time points (Figure 1A-F). Based upon the high levels of cell death in the GM03349 cells at the 100 μM concentration of both carcinogens, we performed subsequent analyses on fixed cells using only 10 μM, 20 μM, and 50 μM of each carcinogen.

Fulvestrant exposure causes a decrease in mitotic index in A549 and UPCI:SCC103 cells, but an increase in GM03349 cells

We exposed our three cell lines to 10 μM, 20 μM, or 50 μM Fulvestrant and fixed samples after 24, 36, and 48 hours. Mitotic indices were then calculated from each sample; each condition was repeated six times, with no less than 500 cells scored per preparation. For A549 cells after 24 hours treatment (Figure 2A), we observed a statistically significant increase in

mitotic index when compared to controls at Fulvestrant concentrations of 10 μM ($p=0.0007$) and 20 μM ($p=0.0005$) , but a significant decrease ($p<0.0001$) at 50 μM Fulvestrant, with a reduction of over half the mitotic index. At 36 hours (Figure 2B), the mitotic index of cells at concentrations of 10 μM and 20 μM were not significantly different, but the mitotic index of cells treated with 50 μM had dropped even further to less than a quarter of the frequency observed in untreated cells. Similar outcomes were observed at 48 hours for 10 μM, with little deviance from the control population and 50 μM where the mitotic index was less than a quarter of that seen in untreated cells, but we also observed a slight but statistically significant drop ($p=0.0355$) at 20 μM Fulvestrant (Figure 2C).

For UPCI:SCC103 cells, we saw an increase in mitotic defect at 10 μM ($p<0.0001$) after 24 hours as well as at 50 μM ($p=0.0202$), but not at 20 μM (Figure 2A). At 36 hours, all three concentrations of Fulvestrant caused a significant decrease in mitotic index, with 50 μM treatment reducing the mitotic index most significantly to less than half of that observed in untreated cells (Figure 2B). This trend continued after 48 hours exposure where we saw statistically-significant, decreases in mitotic indices for all three concentrations ($p<0.0001$ for all three concentrations) of Fulvestrant (Figure 2C).

By contrast, we observed no decrease in mitotic index at any concentration or time of exposure for GM03349. At 24 hours, all three concentrations of Fulvestrant showed a significant increase in mitotic index with 10 μM and 20 μM Fulvestrant nearly doubling the mitotic index and 50 μM Fulvestrant causing a more than doubling of the mitotic index ($p<0.0001$ for all three concentrations). This increase in mitotic index was also observed at 36 hours for all

concentrations (Figure 2B) and again at 48 hours where all three conditions demonstrated mitotic indices more than double what was observed in the untreated sample (Figure 2C).

Vinyl chloride decreases mitotic index in A549 cells after prolonged exposure, but after short exposure in UPCI:SCC103 cells

Next we examined how vinyl chloride would affect the mitotic indices of our cells at varying concentrations over our three timepoints. Cells were treated and processed in the same way as cells exposed to Fulvestrant and scored similarly, with each condition repeated six times. In A549 cells, we noticed no significant difference in mitotic index at 24 hours of exposure for 10 μM ($p=0.5191$) and 20 μM ($p=0.3415$ when compared to untreated cells (Figure 2D), but we did see a slight decrease when the cells were treated with 50 μM ($p=0.349$; Figure 2D). At 36 hours, exposure to both 20 μM ($p=0.0146$) and 50 μM ($p=0.0081$) concentrations of vinyl chloride showed statistically-significant decreases in mitotic index (Figure 2E), and at 48 hours, cells treated with any of the three concentrations of vinyl chloride showed decreased frequency of mitotic cells in the population (10 μM, $p=0.0084$; 20 μM, $p=0.0006$; 50 μM, $p=0.007$; Figure 2F).

In UPCI:SCC103 cells, we saw a stronger effect at all timepoints. At 24 hours, when compared to untreated cells there was a not-quite statistically significant decrease in mitotic index after treatment with 10 μM vinyl chloride ($p=0.0759$), but statistically significant decreases at 20 μM ($p=0.0491$) and 50 μM ($p=0.0193$; Figure 2D). At 36 hours exposure, all three concentrations of vinyl chloride produced statistically significant decreases in mitotic index (10

299 μM, p=0.049; 20 μM, p=0.0226; 50 μM, p=0.004; Figure 2E), and this was also seen to an even
300 greater degree at 48 hours, with each condition exhibiting mitotic indices reduced by at least one
301 third when compared to untreated cells (Figure 2F).

302 Again, with GM03349 cells, we saw the opposite effect: treatment with vinyl chloride
303 increased the mitotic index. This was true for all concentrations at 24 hours, with 10 μM vinyl
304 chloride nearly doubling the mitotic index and 20 μM and 50 μM more than doubling the
305 frequency of mitotic cells in the population compared to untreated cells (Figure 2D). This effect
306 persisted at both 36 hours of vinyl chloride treatment (Figure 2E), as well as after 48 hours of
307 exposure (Figure 2F).

308

309 Exposure to Fulvestrant significantly increases the frequency of mitotic defects

310

311 After having observed changes to the frequency of mitotic cells in our three cell lines, we
312 wished to then examine the frequency at which mitotic defects occurred. For each concentration
313 of Fulvestrant at each timepoint, we counted the number of mitotic cells that were normal or
314 exhibited one of four classifications of mitotic defect: lagging chromosome(s) in
315 prometaphase/metaphase, lagging chromosome(s) in anaphase, the presence of an anaphase
316 bridge or bridges, or a multipolar spindle (Figure 3A-D). Defect indices for each concentration of
317 carcinogen and time point were scored from six different preparations, with 100-150 cells scored
318 per preparation. We observed two broad trends that existed for all three cell lines: a large
319 increase in the frequency of defects in the population, and that lagging chromosomes in
320 prometaphase/metaphase and anaphase bridges were the defects that showed the greatest rates of
321 increase.

A549 cells exhibit a reasonably high level of mitotic defect even when untreated, with approximately 25% of mitotics showing one or more type of defect. Of these cells, the most common defects are lagging chromosomes in anaphase and anaphase bridges accounting for approximately 8% of mitotic cells each; these are approximately twice as prevalent as lagging chromosomes in prometaphase/metaphase and multipolar spindles (Figure 3E; Table 1). When treated with Fulvestrant for 24 hours, we saw an increase of between approximately 10% (10 μM and 20 μM) and 20% (50 μM) of defects in total (Figure 3E). In terms of specific defects, we saw a three-fold (10 μM and 20 μM) to five-fold (50 μM) increase in the percentage of cells with lagging chromosomes in prometaphase/metaphase, a near doubling of the frequency of anaphase bridges, and a small increase in multipolar spindle frequency. By contrast, we saw a decrease in the frequency of lagging chromosomes in anaphase at 10 μM and 20 μM concentrations of Fulvestrant, but not at 50 μM (Table 1). At 36 hours, the frequency of defect was approximately the same for cells treated with 10 μM Fulvestrant, but increased somewhat for 20 μM treatment; 50 μM Fulvestrant treatment led to an even greater increase, with over half of all mitotic cells now exhibiting a defect (Figure 3F). As with 24 hours of exposure, the presence of lagging chromosomes in prometaphase/anaphase were considerably higher, but we saw a decrease in the frequency of anaphase bridges at 50 μM Fulvestrant to below that observed in untreated cells. Additionally, the frequency of multipolar spindles rose two-fold (10 μM and 20 μM) to four-fold (50 μM) compared to untreated cells (Table 1). After 48 hours exposure, we saw another increase in the frequency of defect, with over 40% (10 μM) or over 50% (20 μM and 50 μM) of mitotics possessing defects (Figure 3G; Table 1). Our observations at 48 hours mirrored those at 36 hours: we saw highly-elevated frequencies of mitotic defect (Figure 3G) with large numbers of cells possessing lagging chromosomes in prometaphase/metaphase, as well as increases in the

number of cells with multipolar spindles. Again, while the number of cells with anaphase bridges were elevated in cells treated with 10 μM or 20 μM Fulvestrant, cells treated with 50 μM Fulvestrant showed a decrease in bridge frequency (Table 1).

UPCI:SCC103 cells have a lower number of cells with mitotic defects present in an untreated population than A549 cells, with approximately 14% of mitotics having a defect. Of those, lagging chromosomes in prometaphase/metaphase, lagging chromosomes in anaphase and anaphase bridges are all equally prevalent, with fewer multipolar spindles observed (Figure 3H; Table 1). When we exposed these cells to Fulvestrant for 24 hours, however, the increase in frequency of defect was even more dramatic than observed in A549 cells. Treatment with 10 μM Fulvestrant led to 47.7% of mitotic cells possessing a defect, 20 μM Fulvestrant led to 55.2% of cells exhibiting a defect, and 50 μM Fulvestrant treatment led to 60.1% of mitotic cells possessing a defect (Figure 3H). After 24 hours of treatment, we observed that the frequency of lagging chromosomes increased in the cells from three- to five-fold, anaphase bridges increased between two- to four-fold, multipolar spindle incidence rose by up to eighteen-fold, and lagging chromosomes in anaphase rose by over three-fold in cells treated with 10 μM Fulvestrant although only slightly increased in cells treated with 20 μM Fulvestrant and showed no statistically-significant difference with 50 μM treatment (Table 1). After 36 hours treatment, approximately 60% of all mitotic cells treated with any of the three concentrations of Fulvestrant possessed a defect (Figure 3F). Of those defects, we saw lagging chromosomes in prometaphase/metaphase, anaphase bridges, and multipolar spindles at highly elevated frequencies each accounting for between ~15% and ~20% of total mitotic cells with any of the concentrations of Fulvestrant. Only lagging chromosomes in anaphase appeared unchanged (Table 1). After 48 hours of treatment, the frequency of mitotic defect for all three concentrations

of Fulvestrant was just under 60%, demonstrating no change from cells scored after 36 hours of exposure, but being vastly elevated compared to untreated cells (Figure 3G). Similarly, the frequencies of lagging chromosomes in prometaphase/metaphase, anaphase bridges and multipolar spindles remained elevated, falling between 13 and 20% for each of the specific conditions at each of the three concentrations. Lagging chromosomes in anaphase were far fewer, showing no statistically-significant difference from untreated cells (Table 1).

As a noncancer line, we expected GM03349 cells to show a very low level of mitotic defects, and indeed untreated cells exhibited defects in only 5% of their mitotic population, with multipolar spindles accounting for ~2.5% of those, anaphase bridges accounting for ~1.5%, lagging chromsomes in prometaphase/metaphase ~1%, and lagging chromosomes in anaphase less than 0.5% (Figure 3E; Table 1). As with our two cancer cell lines, 24 hours of treatment with Fulvestrant led to a large increase in the number of defects in the GM03349 mitotic population, rising between three- and five-fold in frequency (Figure 3E). All four scored defects were affected, with large increases in lagging chromosomes in prometaphase/metaphase and anaphase bridges once again. Multipolar spindle frequencies increased at concentrations of 20 μM and 50 μM and lagging chromosomes in anaphase also increased at all three concentrations, particularly at 50 μM, rising to over 6% of all mitotic cells (Table 1). At 36 and 48 hours, there was an even greater prevalence of mitotic defects in the population (Figure 3F-G), with the highest numbers seen (~48%) in cells treated with 50 μM Fulvestrant for 48 hours. The frequency of each of the mitotic defects also remained elevated, with lagging chromosomes in prometaphase/metaphase and anaphase bridges consistently accounting for the majority of defects. While lagging chromosomes in anaphase appeared more prevalent than in the untreated sample, their frequency did not increase beyond those seen after 24 hours. When scoring

multipolar spindles, frequency did not increase beyond those observed in untreated cells for any timepoint with 10 μM Fulvestrant, and appeared to rise slightly and inconsistently with 20 μM treatment. While there was a significant difference between untreated cells and those treated with 50 μM Fulvestrant, this did not appear to be a linear progression, as frequency of multipolar spindles was lower after 36 hours than 24 hours, but higher after 48 hours, being present at a rate over five-fold that seen in untreated cells (Table 1).

Exposure to vinyl chloride increases the frequency of mitotic defects, especially lagging chromosomes in prometaphase/metaphase and anaphase bridges

As with Fulvestrant, we wanted to see the effect of vinyl chloride on the incidence of mitotic defects and the prevalence of each type of defect within the population. We followed the same procedure as described for Fulvestrant, using the same scoring criteria. Each condition was repeated six times, and at least 100 cells were scored per preparation. Broadly speaking, our findings were similar to those observed when cells were treated with Fulvestrant: a large increase in the frequency of mitotic defect in all cell lines

For A549 cells, we observed a significant increase in defect frequency for all three concentrations (10 μM, 20 μM, and 50 μM) at each time point (Figure 3H-J). From the baseline of approximately 25% defect cells, we saw an increase to approximately 40% of mitotic cells with defects at 24 hours exposure for all concentrations, approximately 45% at 36 hours and defect indices of ~50% (10 μM), ~48% (20 μM), and ~54% (50 μM) after 48 hours of exposure. When we counted the specific ratio of defects, at 24 hours, we saw an approximately five-fold increase in the frequency of lagging chromosomes in prometaphase/metaphase at all

concentrations, a doubling in the frequency of anaphase bridges, but no increase in the number of mitotic cells with lagging chromosomes in anaphase or that exhibited multipolar spindles (Table 2). At 36 hours, we observed a slight increase in the frequency of prometaphase/metaphase lagging chromosomes and anaphase bridges beyond the 24 hour timepoint, but we did see at 50 μM vinyl chloride a statistically-significant increase in the number of multipolar spindles within the population as well as a not-quite statistically significant increase in multipolarity at 10 μM. The frequency of lagging chromosomes in anaphase showed a slight reduction from untreated cells with all three concentrations of vinyl chloride (Table 2). Cells fixed after 48 hours continued the trend observed at 36 hours with regards to prometaphase/metaphase lagging chromosomes and anaphase bridges: a moderate increase over the previous timepoint. For multipolar spindles, we observed a significant increase in frequency for all three concentrations, with 10% of mitotic cells being multipolar when they were treated with 50 μM vinyl chloride. The frequency of lagging chromosomes in anaphase also slightly decreased again (Table 2).

As with Fulvestrant treatment, exposure to vinyl chloride strongly increased the frequency of mitotic defects in UPCI:SCC103 cells. Similar to our observations with A549 cells, the frequency of defects in the population rose at each consecutive timepoint when compared to controls for all three concentrations of the carcinogen used (Figure 3H-J). The frequency of specific defects also followed the general patterns we have described. Lagging chromosomes in prometaphase/metaphase increased twofold after 24 hours at any concentration of vinyl chloride used, and threefold at the 36 and 48 hour timepoints. Anaphase bridges also increased steadily at each timepoint, three-fold to four-fold at 24 hours, four-fold to five-fold at 36 hours, and five-fold to nearly seven-fold at 48 hours, with 10 μM vinyl chloride generating the lower end of the range and 50 μM generating the upper end of the frequencies (Table 2). We also observed that

multipolar spindles increased at all times and under all concentrations, with the greatest increases seen for each concentration after 48 hours, and for each timepoint, the 50 μM vinyl chloride treated sample exhibiting the most multipolar spindles (Table 2). For lagging chromosomes in anaphase, we saw no real change under most of the conditions observed, the only conditions under which a statistically significant difference was noted was after 36 hours of treatment with 20 μM vinyl chloride and 48 hours treatment at a 10 μM concentration (Table 2).

Our noncancer cells, GM03349, showed large increases in the frequency of mitotic defect in the population with all concentrations used, at all timepoints. As with the cancer cell lines, the percentage of mitotics with defects rose at each of the three timepoints, and greater concentrations of vinyl chloride produced more defects (Figure 3H-J). As with our observations in the cancer cell lines, increases in frequencies of prometaphase/metaphase lagging chromosomes and anaphase bridges accounted for much of this increase, with each timepoint exhibiting more of each defect, and treatment with 50 μM vinyl chloride generating the most of either defect (Table 2). We also saw that multipolar spindles increased in frequency: cells fixed after 24 hours showed an increase when treated with 50 μM vinyl chloride, but not 10 μM or 20 μM; however, with 36 and 48 hours of exposure, all three concentrations generated significant increases in the frequency of multipolarity (Table 2). Anaphase lagging chromosome frequency also generally rose with the increase in exposure time and concentration: only 10 μM treatment for 24 and 48 hours as well as 50 μM treatment for 48 hours failed to exhibit significant increases in their prevalence (Table 2).

Cells with lagging chromosomes in prometaphase/metaphase are less likely to complete mitosis.

Having observed significant increases in the frequency of lagging chromosomes in prometaphase/metaphase as well as anaphase bridges in response to treatment with our carcinogens, we wanted to determine the fate of these cells via live cell imaging. We used SiR-DNA to fluorescently label DNA in each of the three cell lines and examined mitotic progression in untreated cells as well as cells treated with 20 μM vinyl chloride for 24 hours prior to imaging. In untreated A549 cells, all cells in which we observed no mitotic defects (n=7) completed mitosis normally, as did the cells in which we saw anaphase bridges (n=2) and lagging chromosomes in anaphase (n=1). One cell had a lagging chromosome in prometaphase/metaphase and did not complete mitosis. For untreated UPCI:SCC103 cells, seven of eight cells showing no defects completed mitosis normally, as did the cells with anaphase bridges (n=2) and lagging chromosomes in anaphase (n=1); a cell with a lagging chromosome in prometaphase/metaphase did not complete mitosis. In GM03349 cells, the majority imaged contained no defect and completed mitosis (n=9), with the only defect observed being a cell with an anaphase bridge, which completed mitosis.

For cells that had been exposed to 20 μM vinyl chloride, we observed higher rates of defects and a higher frequency of failure of mitosis. In A549 cells, 44% of cells completed mitosis while 56% (n=54) failed to do so. Seventeen cells showed no obvious defects and eleven of these completed mitosis normally (Figure 4A, Movie S1), while six did not. Twenty-two cells exhibited a lagging chromosome in prometaphase/metaphase (Figure 4B, Movie S2) and only four completed mitosis. We also observed sixteen cells with anaphase bridges, nine of which completed mitosis (Movie S3). Of the seven cells with anaphase bridges that failed to complete

mitosis, most (6/7) seemed to progress fully through anaphase before the chromosomes collapsed back into a single mass. One cell showed an anaphase lagging chromosome and completed mitosis normally.

In treated UPCI:SCC103 cells, only 31% of cells successfully completed mitosis while 69% did not (n=39). Of cells with no obvious defect, four completed mitosis and six did not. Interestingly, one cell that failed to complete mitosis collapsed prior to anaphase and ejected chromosomal material from its main mass (Figure 4C, Movie S4). Twenty-one cells had a prometaphase/metaphase lagging chromosome, only five of which completed mitosis while seventeen failed (Figure 4D, Movie S5). Two cells exhibited a lagging chromosome in anaphase, both of which completed mitosis, and nine cells exhibited anaphase bridges, with five cells completing mitosis (Figure 4E, Movie S6) and four failing to do so. We also saw one cell with a multipolar spindle, which collapsed and failed to divide.

The success rate of mitosis in GM03349 cells treated with vinyl chloride was 50% (n=26). Thirteen cells exhibited no defects, and nine of these completed mitosis. We identified eight cells with a prometaphase/metaphase lagging chromosome, and saw that two of these completed mitosis, while six did not. Eight cells had anaphase bridges; half completed mitosis and half did not.

Defect frequency, but not mitotic index, normalizes after vinyl chloride washout

After determining the strong effects of the carcinogens on our cells in terms of prevalence of mitotic defects and observing the heavy increase in the frequency of mitotic failure in vinyl chloride treated cells, we wanted to see if or how the cells would recover after the vinyl chloride

was removed from the environment. We treated all three cell lines with 20 μM vinyl chloride for 24 hours, then washed it out with normal media, and fixed cells after 0, 24, 48, 72, and 96 hours. Cells were then scored for mitotic index, defect index, and types of defects observed. As previously observed, 24 hour treatment with 20 μM vinyl chloride had only a minor effect on mitotic index in A549 cells, but did cause a significant decrease in mitotic index in UPCI:SCC103 cells and a significant increase in mitotic index in GM03349 cells. After 24 hours of washout, however, we saw that the mitotic index in both A549 and UPCI:SCC103 was lower still. This trend continued, with a statistically significant decrease in A549 cells evident by 72 hours post-washout. In GM03349 cells, the high mitotic index persisted for 24 hours after washout, but then dropped, returning to normal levels after 72 hours of washout (Figure 5).

When we scored defect frequencies in our cell lines, we observed the same trend in all three cell lines. As before, we observed a large increase in defect frequency after 24 hours of treatment with 20 μM vinyl chloride, but then observed a steady decline in the frequency of defect and by 96 hours post washout, the number of defects observed was at or near baseline (untreated) frequencies (Figure 6). Individual defect frequencies also returned to normal or near-normal frequencies as the timecourse progressed (Table 3).

Discussion

Our findings here show that brief exposure to two different carcinogenic molecules is sufficient to drive large-scale changes in mitotic progression and mitotic outcomes. Both cancer and noncancer cells show changes in the frequency of mitotic defects and exhibit similar patterns of failure to complete mitosis.

After having determined optimal concentrations of Fulvestrant and vinyl chloride to administer to our cells, we saw that mitotic index in each cell line was affected (Figure 2). In GM03349 cells, we expected and observed an increase in the mitotic index. This suggests that the carcinogens are causing sufficient DNA or spindle damage to induce an arrest in these dividing cells; a higher mitotic index is indicative of cells spending a greater duration of time in mitosis. However, our cancer cells did not yield the same result. For A549 cells, we saw that there was an increase in mitotic index after 24 hours with our two lower concentrations of Fulvestrant (10 μM and 20 μM), but this did not persist at 36 hours or 48 hours. We also saw a decrease in the mitotic index when cells were treated with 50 μM Fulvestrant. This suggests that while the lowest doses for the shortest times might lead to an arrest as observed in the noncancer line, cells fail to enter or exit early from mitosis at the higher concentration, increasing in frequency within the population over time of exposure. When A549 cells were treated with vinyl chloride, we observed a decline in mitotic index across all three concentrations by 36 hours of treatment. For UPCI:SCC103 cells, we saw the same pattern for each carcinogen: a steady decrease in mitotic index that was both time- and concentration-dependent. At the highest concentrations of either carcinogen and longest times of exposure, our two cancer lines exhibited a similar decrease in mitotic index, suggesting a common mechanism of failure, but UPCI:SCC103 cells may be more susceptible to activation of this failure.

We next scored each of our cell lines for mitotic defects under the various conditions of time and concentration of carcinogen exposure. Regardless of concentration or time of exposure, we saw a massive increase in the frequency of mitotic defects (Figure 3). Of our two cancer cell lines, UPCI:SCC103 appeared more sensitive to Fulvestrant, which correlates with our observation of the different pattern in mitotic index. While A549 and GM03349 cells appeared to

552 have a dose-dependent response to Fulvestrant at each of our timepoints, UPCI:SCC103 cells
553 were fairly uniform in the frequency of defects after 36 hours at any concentration. Cells treated
554 with vinyl chloride showed something of a more expected response: 50 μM treatment producing
555 the most defects in each cell line at each time point.

556 When we looked at individual mitotic defect frequencies we saw a number of patterns
557 that were consistent across all three cell lines and with either carcinogen (Table 1, Table 2).
558 Prometaphase/metaphase lagging chromosomes increased in frequency at each subsequent
559 timepoint, and higher concentrations of carcinogen yielded greater numbers of lagging
560 chromosomes prior to anaphase. We also saw a similar pattern with anaphase bridges: all three
561 cell lines showed a large increase after 24 hours of exposure that increased further at the later
562 timepoints. These two mitotic defects accounted for the majority of those induced by carcinogen
563 treatment. It is interesting to note that the two carcinogens, which harm the cell in different ways,
564 induced increases in the likelihood of the same mitotic defects occurring. We did not see large
565 increases in the frequency of anaphase lagging chromosomes. While we did see an increase early
566 in the timecourse with UPCI:SCC103 cells at lower concentrations of Fulvestrant (10 μM and 20
567 μM) and at higher Fulvestrant concentrations in GM03349 (50 μM), these did not persist through
568 the remaining timepoints. When treated with vinyl chloride, a moderate increase was observed in
569 GM03349 and UPCI:SCC103 cells, but overall we did not see dramatic increases in the
570 frequency of their occurrence. Multipolar spindle frequency increased after carcinogen exposure,
571 but there did appear to be a slight lag time to any large-scale increase in their frequency when
572 compared to that seen with prometaphase/metaphase lagging chromosomes and anaphase
573 bridges. This may be due to there being more requirements for a multipolar spindle to be formed.
574 Previous work has demonstrated that multipolar spindles require both the presence of extra

centrosomes as well as an additional change that prevents the cells from clustering the centrosomes (Ganem et al. 2009; Kwon et al. 2008; Quintyne et al. 2005); so as the timecourse progresses it is possible that the events that will lead to changes in centrosome number (i.e. failure of cytokinesis, overduplication of centrosomes, or decoupling of the DNA replication cycle from the centrosome duplication cycle (Brinkley 2001; Nigg 2002)) are increasing. These changes then lead to a higher rate of multipolarity later on.

To better understand the fate of the carcinogen-treated cells, we used live cell imaging to follow progression through mitosis. There was a low frequency (~10%) of mitotic failure in our untreated cells, as well as a low frequency of mitotic defects in the A549 and UPCI:SCC103 cells. Treatment with 20 μM vinyl chloride for 24 hours had profound effects upon both of these phenomena. First, we saw a much higher frequency of mitotic failure, accounting for 50% of noncancer cells and in excess of 50% for the cancer cells, demonstrating that the vinyl chloride had a heavy impact on the cells' ability to complete mitosis successfully. Second we saw more cells exhibiting mitotic defects as expected, but we also observed that there were correlations between the type of defect observed and the success rate at mitotic completion. Of cells with a lagging chromosome in prometaphase/metaphase, over 80% of A549 cells, over 75% of UPCI:SCC103 cells, and 75% of GM03349 cells failed to complete mitosis. By comparison, lagging chromosomes in anaphase were rare (A549: 1/54; UPCI:SCC103: 2/39), but always completed mitosis. Previous work has established that lagging chromosomes are a key contributor to chromosomal instability via mis-segregation and there has been an increasing body of evidence linking merotelic spindle-kinetochore attachments to the prevalence of lagging chromosomes, although this analysis has focused on lagging chromosomes in anaphase (Bakhoum et al. 2014; Cimini et al. 2004; Cimini et al. 2001; Cimini et al. 2003; Ganem et al.

2009; Thompson & Compton 2011). It has been proposed that the DNA damage response during mitosis as well as pre-mitotic replicative damage carried forward into mitosis increases the frequency of lagging chromosomes (Bakhoum et al. 2014; Burrell et al. 2013). Error correction mechanisms have also been proposed for the resolution of merotelic connections (Cimini et al. 2003). Our results suggest that these merotelic connections, as they form in prometaphase, can be sufficient to prevent progress past the metaphase-anaphase transition, and a cell that is caught there may simply exit mitosis. Given that vinyl chloride will affect a cell by causing DNA damage and altering topoisomerase function, it is likely that damage to DNA prior to, and during, mitosis contributes to this mitotic exit. That we saw no failure of division in our small number of cells with anaphase lagging chromosomes suggests that the ability to abort mitosis due to the merotelic connections is lost after the cell progresses into anaphase.

For cells with anaphase bridges, we observed that slightly under half of cancer cells and half of noncancer cells subsequently failed to complete mitosis. In some cases, we observed the two masses of chromatin separate from each other and appear to have entered telophase before the two sets of chromosomes collapsed back towards each other. Our observations are consistent with the previously described phenomenon that only some chromosome bridges break, while many persist into the subsequent cell cycle stage (Pampalona et al. 2016). We only found one multipolar spindle in all of our acquisitions, and this cell failed to complete mitosis. This may be due to only exposing the cells to vinyl chloride to the shortest of our time intervals, but also may reflect the transitory state of a multipolar spindle as has previously been proposed (Ganem et al. 2009): multipolars only exist as an intermediate state prior to centrosome clustering which may have been less clear in our live cells than fixed samples. We also saw some cells in which no obvious defects were present that failed to complete mitosis, and in several cases appeared to

eject chromatin from the main mass. These may be the result of "lagging" chromosomes that are buried (i.e. they maintain merotelic connections but are spatially oriented within the other chromosomes), and the ejection of this material may be due to unequal polar ejection forces produced by an unresolved merotelic connection.

When we examined the state of cells after washout of vinyl chloride, we expected to see the cells return to a baseline state. This was observed in GM03349 cells, where both mitotic index and defect frequency after 96 hours of washout were indistinguishable from frequencies observed in untreated cells. However, in both A549 and UPCI:SCC103 cells, we saw that while the defect frequency observed in those cells steadily decreased (Figure 6) matching those seen in untreated cells after 96 hours of treatment, the mitotic index did not return to normal levels (Figure 5). For both cell lines, we observed a progressive decrease in the mitotic index at subsequent timepoints after washout. This suggests that there is an increasing proportion of the cell population that has been removed from the proliferative cycle. Such observations were recorded when extra centrosomes were artificially generated in cells (Ganem et al. 2009), and we propose that a similar process is occurring here: as cells exit mitosis early due to unresolvable lagging chromosomes, they are arrested and do not re-enter mitosis.

Conclusions.

In summary, we demonstrate here that brief exposure to two different types of carcinogenic molecule is sufficient to induce large increases in mitotic defects in both cancer and noncancer cell lines. Furthermore, we observe that lagging chromosomes in prometaphase/metaphase and anaphase bridges are especially prevalent, and in both cases the

644 occurrence of this defect leads to a high probability that the cell will fail to complete mitosis. By
645 comparison, lagging chromosomes in anaphase do not appear to impede completion of mitosis.
646 Additionally, we see that upon washout of vinyl chloride, defect incidences revert back to normal
647 levels but mitotic index does not in either of our cancer lines, suggesting a proportion of treated
648 and washed-out cells have been removed from the proliferative cycle. Taken together, our
649 findings here show that there are significant short- and longer-term effects on the replicative state
650 of a population of cancer cells from a single exposure to carcinogen.

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831

Figure 1

Cell viability after treatment with Fulvestrant or vinyl chloride.

A549, UPCI:SCC103, and GM03349 cells were treated with 10 µM, 20 µM, 50 µM, or 100 µM Fulvestrant for (A) 24 hours, (B) 36 hours, or (C) 48 hours prior to being harvested and a viability count performed using ViaCount reagent for flow cytometry. Cells were also treated with 10 µM, 20 µM, 50 µM, or 100 µM vinyl chloride for (D) 24 hours, (E) 36 hours, or (F) 48 hours prior to a viability count. Each condition was repeated three times. A student t-test was performed to compare untreated cells to each concentration/time. Conditions that exhibit a statistically significant difference from untreated cells are indicated: *=p<0.05, **=p<0.01. Full p-values for all conditions are listed in the supplementary raw data files.

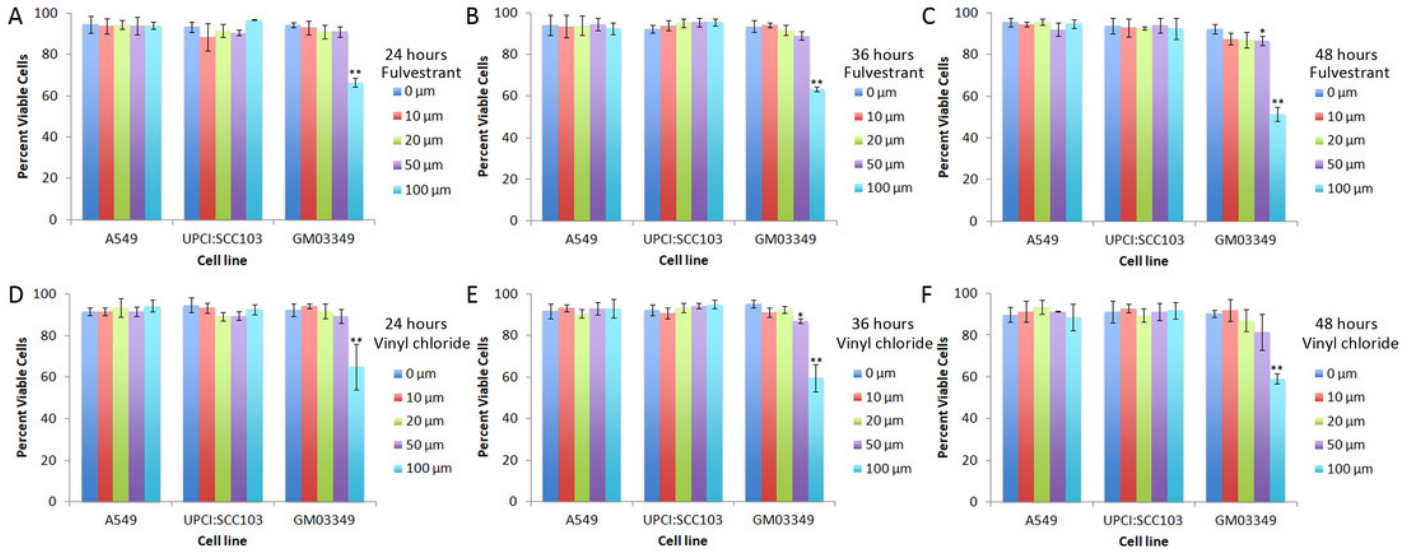

Figure 2

Mitotic index after treatment with Fulvestrant or vinyl chloride.

A549, UPCI:SCC103, and GM03349 cells were seeded on coverslips and then treated with 10 µM, 20 µM, or 50 µM Fulvestrant for (A) 24 hours, (B) 36 hours, or (C) 48 hours prior to being fixed and stained with DAPI. Cells were also treated with 10 µM, 20 µM, or 50 µM vinyl chloride for (D) 24 hours, (E) 36 hours, or (F) 48 hours prior to fixation and DAPI staining. Slides were scored for mitotic index, with at least 500 cells scored per slide. Each condition was repeated six times A student t-test was performed to compare the mitotic index of untreated cells to each concentration/time. Conditions that exhibit a statistically significant difference from untreated cells are indicated: *=p<0.05, **=p<0.01. Full p-values for all conditions are listed in the supplementary raw data files.

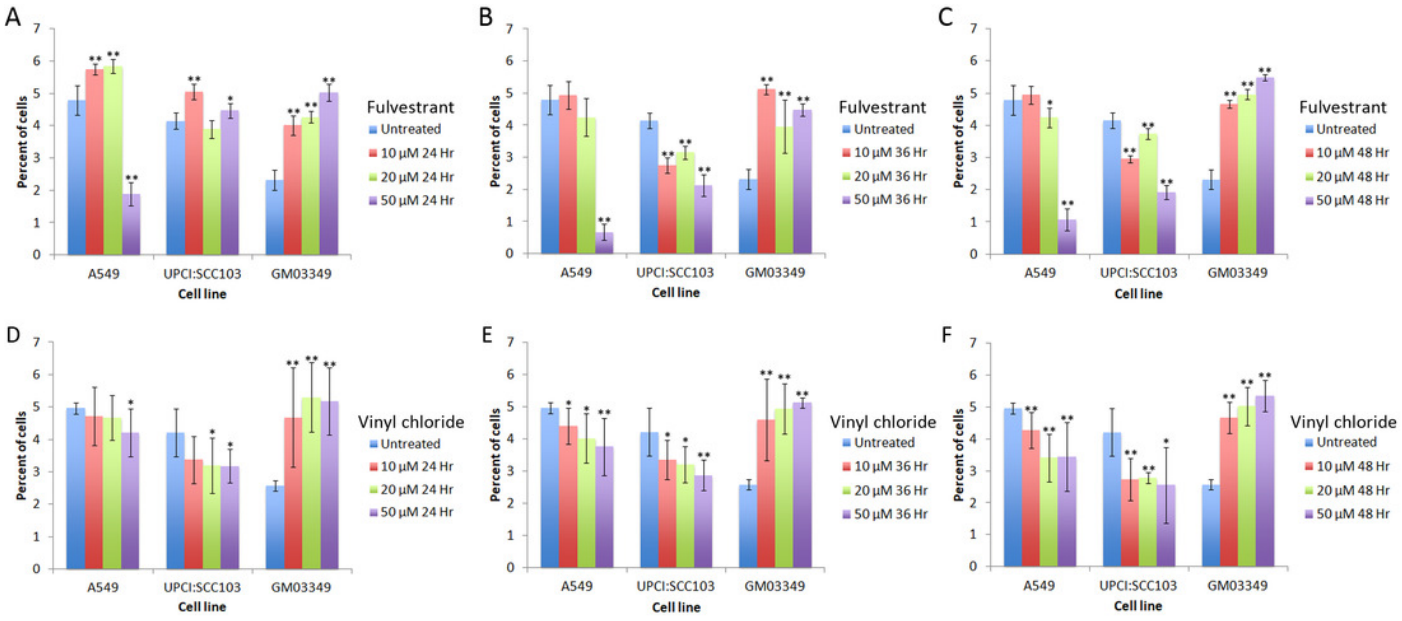

Figure 3

Defect frequency after treatment with Fulvestrant or vinyl chloride.

Treated A549, UPCI:SCC103, and GM03349 cells that had been fixed and stained with DAPI were scored for mitotic defects. The defects scored were (A) Lagging chromosomes in prometaphase or metaphase, (B) lagging chromosomes in anaphase, (C) anaphase bridges, and (D) multipolar spindles. Bar = 10 µm in all images. Each cell line was scored for overall defect frequency at 10 µM, 20 µM, or 50 µM Fulvestrant treatment after (E) 24 hours exposure, (F) 36 hours exposure, and (G) 48 hours exposure. Each cell line was also scored for overall defect frequency at 10 µM, 20 µM, or 50 µM vinyl chloride treatment after (H) 24 hours exposure, (I) 36 hours exposure, and (J) 48 hours exposure. Cells were scored for presence of defects, each condition was repeated six times. A student t-test was performed to compare the mitotic index of untreated cells to each concentration/time. Conditions that exhibit a statistically significant difference from untreated cells are indicated: **=$p<0.01$. Full p-values for all conditions are listed in the supplementary raw data files.

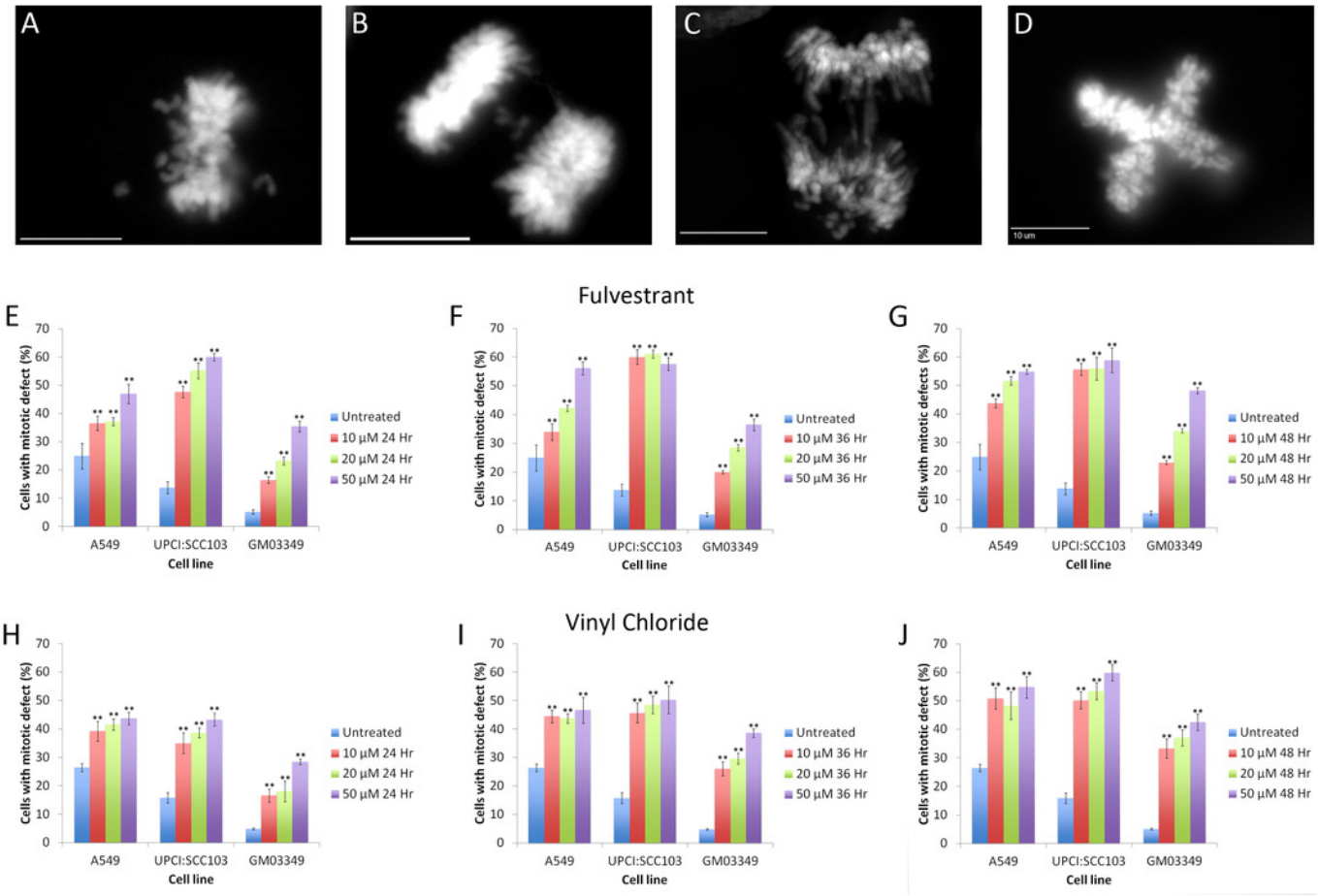

Figure 4

Live cell analysis of mitotic progression.

A549, UPCI:SCC103 or GM03349 cells were seeded on glass bottom petri dishes and treated for 24 hours with 20 µM vinyl chloride. Cells were then treated with SiR-DNA (far red-shifted Hoechst) stain for 30 minutes, and imaged on the confocal microscope for 8+ hours. (A) Stills from movie S1; A549 cell with no clear defects completing mitosis, images are acquisitions at (hh:mm) 00:00, 00:12, 00:24, 00:33, 00:42, and 01:18. (B) Stills from movie S2, A549 cell with a prometaphase lagging chromosome that fails to proceed to anaphase and exits mitosis; images are acquisitions at (hh:mm) 00:00, 00:06, 00:18, 00:33, 01:00, 01:45. (C) Stills from movie S4, UPCI:SCC103 cell with no obvious mitotic defects fails to progress to anaphase and ejects chromatin as it fails to complete mitosis; images are acquisitions at (hh:mm) 00:00. 00:06, 00:39, 01:27, 01:42, 01:54. (D) Stills from movie S5, UPCI:SCC103 cell with prometaphase/metaphase lagging chromosome that subsequently fails to divide; images are acquisitions at (hh:mm) 00:54, 01:21, 01:27, 01:54, 02:06, 02:27. (E) Stills from movie S6, UPCI:SCC103 cells with anaphase bridges; images are acquisitions at (hh:mm) 00:00, 00:12, 00:18, 00:21, 00:39, 00:54. Bar = 5 µm in all images

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

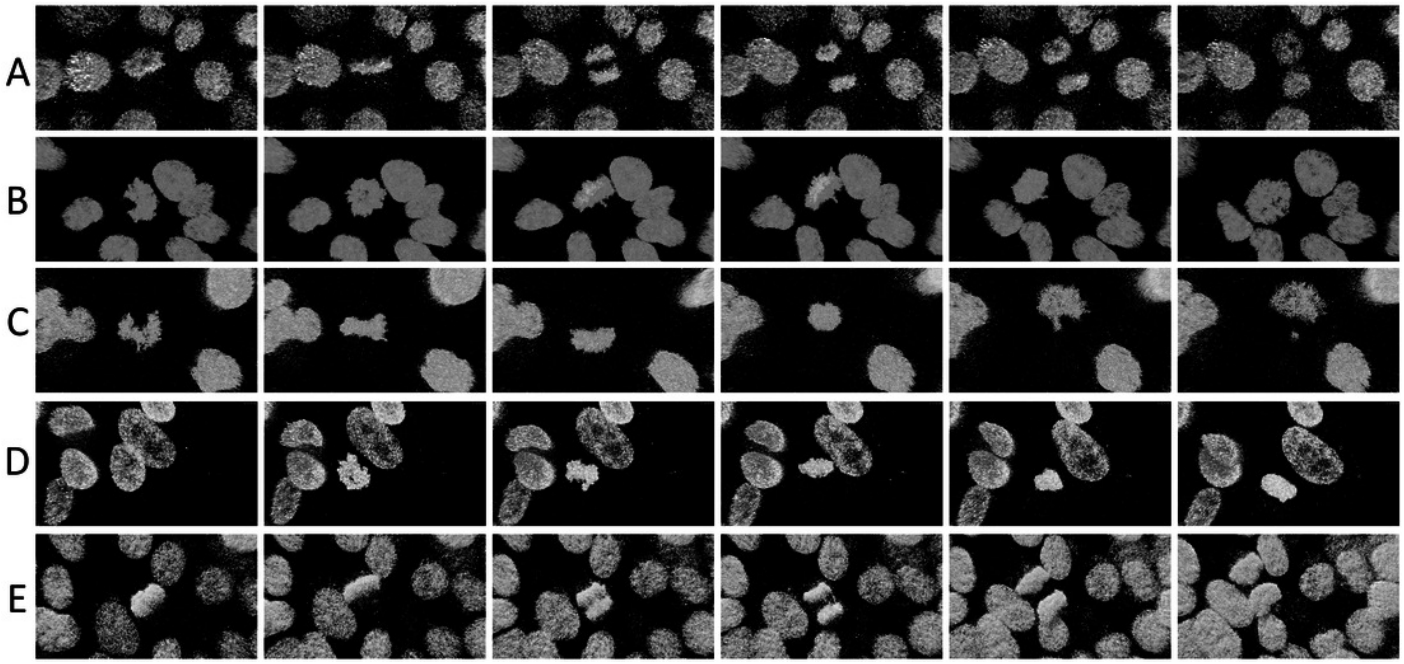

Figure 5

Mitotic index after vinyl chloride treatment and washout.

A549, UPCI:SCC103 and GM03349 cells were treated with 20 µM vinyl chloride for 24 hours before washout with fresh media. Cells were fixed at 0, 24, 48, 72, and 96 hours post-washout and stained with DAPI. At least 500 cells per slide were scored for mitotic index. Each timepoint was repeated six times. A student t-test was performed to compare the mitotic index of untreated cells to each concentration/time. Conditions that exhibit a statistically significant difference from untreated cells are indicated: *=p<0.05, **=p<0.01. Full p-values for all conditions are listed in the supplementary raw data files.

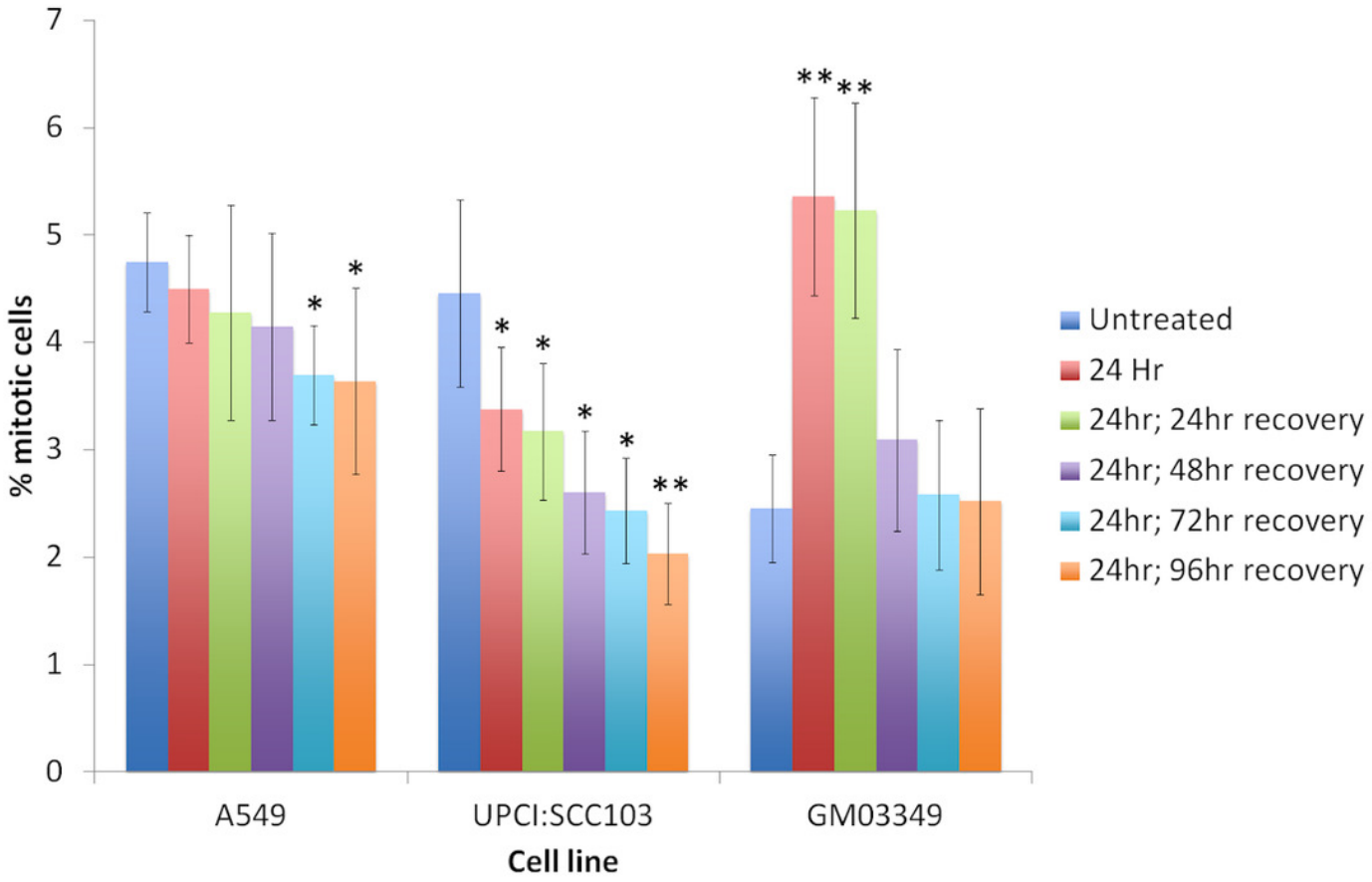

Figure 6

Defect frequency after vinyl chloride treatment and washout.

A549, UPCI:SCC103 and GM03349 cells were treated with 20 µM vinyl chloride for 24 hours before washout with fresh media. Cells were fixed at 0, 24, 48, 72, and 96 hours post-washout and stained with DAPI. At least 100 cells per slide were scored for mitotic defects. Each timepoint was repeated six times. A student t-test was performed to compare the mitotic index of untreated cells to each concentration/time. Conditions that exhibit a statistically significant difference from untreated cells are indicated: *=p<0.05, **=p<0.01. Full p-values for all conditions are listed in the supplementary raw data files.

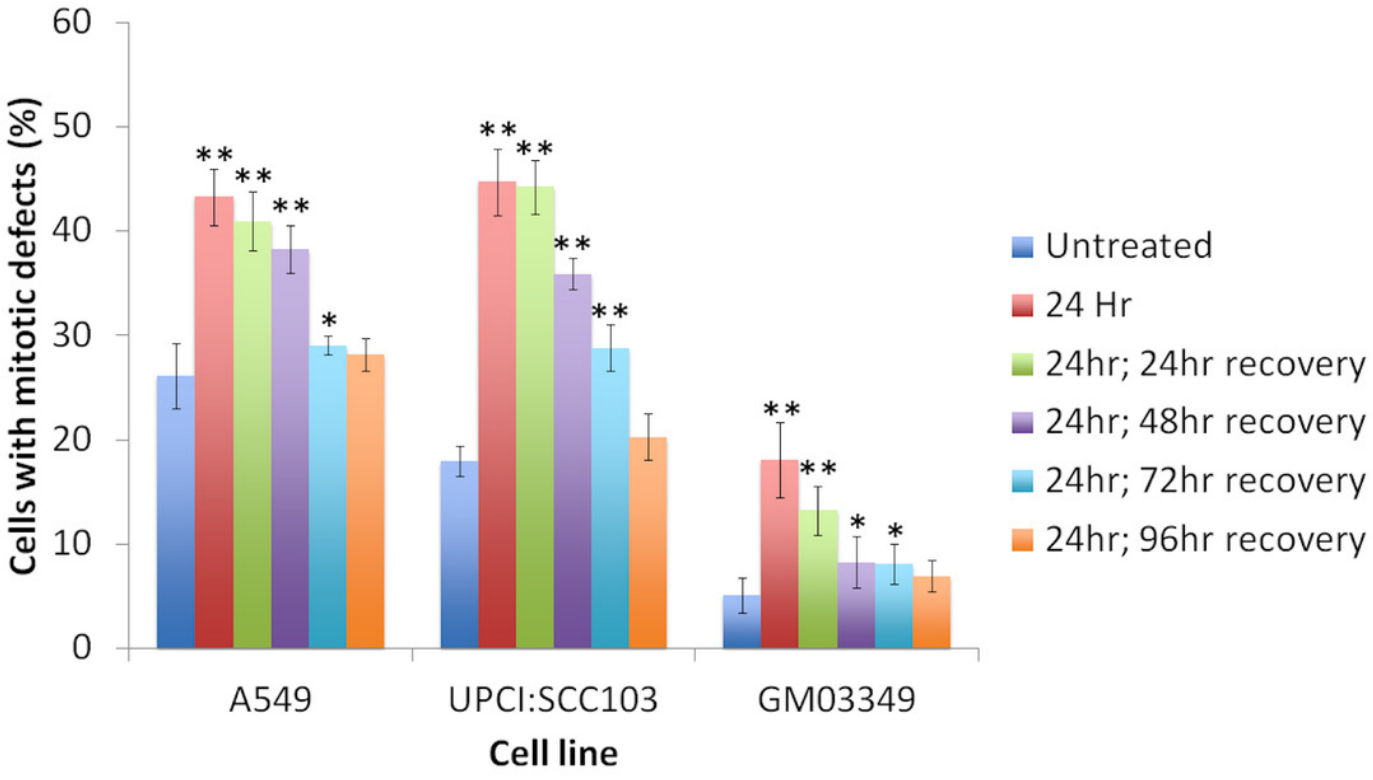

Table 1(on next page)

Frequency of mitotic defects after treatment with Fulvestrant.

A549, UPCI:SCC103, and GM03349 cells were seeded on coverslips and then treated with 10 μM, 20 μM, or 50 μM Fulvestrant for 24 hours, 36 hours, or 48 hours prior to being fixed and stained with DAPI. Mitotic cells were scored for each type of mitotic defects. Numbers represent mean ± standard deviation for counts from six independent trials.

1

Cell Line	Condition	Lagging Chromsomes Prometaphase /Metaphase	Lagging Chromosomes Anaphase	Anaphase Bridges	Multipolar Spindles
	Untreated	4.68 ± 1.45	8.44 ± 3.36	7.65 ± 3.13	4.20 ± 2.42
	10 μM 24 hr	13.71 ± 2.52	4.56 ± 1.38	11.66 ± 1.08	6.71 ± 1.39
	10 μM 36 hr	10.83 ± 1.95	3.26 ± 0.69	11.34 ± 2.05	8.56 ± 1.58
	10 μM 48 hr	16.34 ± 2.38	1.56 ± 1.04	16.48 ± 1.04	9.41 ± 1.56
A549	20 μM 24 hr	13.70 ± 2.19	3.63 ± 1.31	11.30 ± 1.89	8.60 ± 1.38
	20 μM 36 hr	13.75 ± 0.88	8.56 ± 2.03	9.73 ± 2.47	10.18 ± 1.30
	20 μM 48 hr	15.91 ± 2.12	3.35 ± 1.20	19.75 ± 2.19	12.68 ± 1.32
	50 μM 24 hr	22.59 ± 1.83	7.29 ± 1.49	11.75 ± 2.74	5.34 ± 1.26
	50 μM 36hr	28.53 ± 4.29	3.25 ± 3.13	5.64 ± 1.98	18.68 ± 2.29
	50 μM 48 hr	26.74 ± 1.49	4.74 ± 1.31	3.94 ± 1.55	19.40 ± 1.98
	Untreated	4.03 ± 0.65	3.05 ± 1.34	5.11 ± 1.09	1.64 ± 0.99
	10 μM 24 hr	12.10 ± 1.41	11.32 ± 0.83	11.59 ± 2.17	12.70 ± 1.56
	10 μM 36 hr	18.35 ± 2.59	4.40 ± 1.40	23.04 ± 2.18	14.24 ± 1.13
	10 μM 48 hr	15.94 ± 2.27	5.01 ± 2.41	18.02 ± 2.36	16.71 ± 2.28
UPCI:SCC103	20 μM 24 hr	17.79 ± 1.60	5.90 ± 0.87	14.39 ± 1.42	17.13 ± 1.37
	20 μM 36 hr	16.65 ± 1.70	3.80 ± 1.59	20.28 ± 2.01	20.57 ± 1.23
	20 μM 48 hr	18.18 ± 1.41	2.18 ± 1.85	16.23 ± 1.82	19.32 ± 2.96
	50 μM 24 hr	19.95 ± 0.86	2.24 ± 1.00	19.41 ± 1.14	18.47 ± 1.05
	50 μM 36hr	17.77 ± 1.97	2.46 ± 1.89	17.74 ± 1.59	19.71 ± 2.71
	50 μM 48 hr	13.67 ± 1.17	3.45 ± 1.98	17.61 ± 2.60	24.18 ± 1.7
	Untreated	1.08 ± 0.37	0.25 ± 0.27	1.41 ± 0.36	2.50 ± 0.56
	10 μM 24 hr	4.88 ± 0.50	2.01 ± 0.72	6.56 ± 0.74	3.08 ± 0.52
	10 μM 36 hr	8.72 ± 0.45	1.79 ± 0.55	7.01 ± 0.70	2.46 ± 0.54
	10 μM 48 hr	9.44 ± 0.89	1.82 ± 1.08	8.38 ± 1.90	3.36 ± 1.35
GM03349	20 μM 24 hr	6.11 ± 1.05	1.80 ± 1.00	7.80 ± 0.74	7.59 ± 1.15
	20 μM 36 hr	12.65 ± 1.29	3.04 ± 1.00	9.94 ± 0.87	2.86 ± 1.05
	20 μM 48 hr	12.82 ± 1.50	2.10 ± 0.47	13.43 ± 2.55	5.73 ± 1.01
	50 μM 24 hr	9.21 ± 1.27	6.45 ± 0.56	10.89 ± 1.09	9.97 ± 0.83
	50 μM 36hr	13.74 ± 1.09	2.59 ± 0.67	14.10 ± 0.77	6.03 ± 0.91
	50 μM 48 hr	14.40 ± 0.99	5.85 ± 0.27	14.07 ± 1.28	13.96 ± 1.44

2

Table 2 (on next page)

Mitotic defect frequency after treatment with vinyl chloride.

A549, UPCI:SCC103, and GM03349 cells were seeded on coverslips and then treated with 10 μM, 20 μM, or 50 μM vinyl chloride for 24 hours, 36 hours, or 48 hours prior to being fixed and stained with DAPI. Mitotic cells were scored for each type of mitotic defects. Numbers represent mean ± standard deviation for counts from six independent trials.

1

Cell Line	Condition	Lagging Chromsomes Prometaphase /Metaphase	Lagging Chromosomes Anaphase	Anaphase Bridges	Multipolar Spindles
	Untreated	2.74 ± 0.54	9.69 ± 1.57	7.77 ± 1.15	6.29 ± 0.89
	10 μM 24 hr	13.48 ± 1.59	7.56 ± 1.98	12.89 ± 2.41	5.31 ± 1.95
	10 μM 36 hr	13.03 ± 2.27	8.14 ± 1.53	15.01 ± 1.33	8.31 ± 2.23
	10 μM 48 hr	17.19 ± 2.80	6.55 ± 1.01	17.18 ± 1.37	9.31 ± 2.15
A549	20 μM 24 hr	14.29 ± 1.58	7.84 ± 1.84	14.81 ± 1.53	4.67 ± 2.14
	20 μM 36 hr	13.55 ± 3.21	7.15 ± 0.91	15.93 ± 1.71	7.19 ± 1.98
	20 μM 48 hr	17.37 ± 3.11	6.51 ± 1.20	14.82 ± 3.92	9.55 ± 2.20
	50 μM 24 hr	14.42 ± 3.02	7.74 ± 0.87	16.94 ± 2.73	4.62 ± 2.46
	50 μM 36hr	18.64 ± 3.90	7.53 ± 0.94	18.41 ± 6.40	7.57 ± 0.72
	50 μM 48 hr	21.86 ± 3.88	6.60 ± 1.10	16.10 ± 3.64	10.23 ± 1.47
	Untreated	7.15 ± 1.12	0.99 ± 0.83	3.70 ± 0.93	4.02 ± 0.91
	10 μM 24 hr	14.01 ± 2.19	2.08 ± 0.90	10.81 ± 2.62	8.13 ± 2.07
	10 μM 36 hr	20.13 ± 1.68	1.76 ± 1.31	13.95 ± 2.81	9.90 ± 1.91
	10 μM 48 hr	17.60 ± 2.24	2.40 ± 1.23	18.82 ± 3.12	11.29 ± 1.31
UPCI:SCC103	20 μM 24 hr	15.11 ± 2.73	2.42 ± 1.34	13.01 ± 2.03	8.09 ± 2.69
	20 μM 36 hr	19.19 ± 2.66	3.18 ± 1.20	15.07 ± 3.36	11.15 ± 1.59
	20 μM 48 hr	19.86 ± 1.85	1.97 ± 1.02	20.28 ± 1.87	11.25 ± 1.96
	50 μM 24 hr	18.48 ±1.50	1.02 ± 0.82	14.15 ± 2.18	9.65 ± 2.55
	50 μM 36hr	21.04 ± 2.56	2.16 ± 1.53	15.32 ± 2.34	11.81 ± 1.70
	50 μM 48 hr	21.06 ± 2.65	1.75 ± 1.20	20.98 ± 1.92	16.01 ± 3.45
	Untreated	1.33 ± 0.57	0.68 ± 0.46	0.86 ± 0.30	2.01 ± 0.33
	10 μM 24 hr	5.38 ± 1.21	1.93 ± 1.54	7.31 ± 2.44	2.00 ± 1.46
	10 μM 36 hr	10.19 ± 1.99	2.68 ± 1.48	8.95 ± 2.96	4.30 ± 1.57
	10 μM 48 hr	12.98 ± 2.99	1.77 ± 1.51	9.68 ± 2.86	8.76 ± 1.28
GM03349	20 μM 24 hr	4.91 ± 2.52	2.58 ± 1.13	6.46 ± 2.07	4.12 ± 3.17
	20 μM 36 hr	12.31 ± 2.11	1.87 ± 1.22	10.73 ± 1.83	4.81 ± 1.75
	20 μM 48 hr	14.79 ± 2.08	2.09 ± 0.82	13.55 ± 2.28	6.52 ± 1.32
	50 μM 24 hr	8.31 ± 1.89	2.79 ± 2.18	11.13 ± 2.34	6.24 ± 2.25
	50 μM 36hr	15.21 ± 2.25	3.30 ± 1.72	13.74 ± 2.28	6.44 ± 2.02
	50 μM 48 hr	17.70 ± 1.71	2.20 ± 1.83	14.61 ± 1.98	7.89 ± 1.28

2

Table 3(on next page)

Mitotic defect frequency after treatment with 20 μM vinyl chloride followed by washout.

A549, UPCI:SCC103 and GM03349 cells were treated with 20 μM vinyl chloride for 24 hours before washout with fresh media. Cells were fixed at 0, 24, 48, 72, and 96 hours post-washout and stained with DAPI. Mitotic cells were scored for each type of mitotic defects. Numbers represent mean ± standard deviation for counts from six independent trials.

1

Cell Line	Treatment with 20 μM vinyl chloride	Lagging Chromsomes Prometaphase /Metaphase	Lagging Chromosomes Anaphase	Anaphase Bridges	Multipolar Spindles
	no treatment	3.53 ± 1.40	7.93 ± 1.03	9.14 ± 1.25	5.47 ± 1.45
	24 hr; 0 washout	15.04 ± 2.10	8.56 ± 0.89	13.99 ± 1.60	5.68 ± 0.58
A549	24 hr; 24 hr washout	15.49 ± 2.07	7.52 ± 1.71	11.44 ± 0.78	6.49 ± 1.65
	24 hr; 48 hr washout	13.40 ± 2.23	7.85 ± 1.21	10.46 ± 1.65	6.50 ± 1.77
	24 hr; 72 hr washout	7.34 ± 1.04	9.07 ± 1.22	6.55 ± 1.44	6.10 ± 1.30
	24 hr; 96 hr washout	6.88 ± 0.07	7.52 ± 1.19	7.53 ± 1.74	6.19 ± 1.65
	no treatment	6.26 ± 1.49	1.24 ± 1.06	5.62 ± 0.93	4.81 ± 0.80
	24 hr; 0 washout	16.84 ± 1.66	2.71 ± 1.09	15.84 ± 4.24	9.34 ± 1.41
UPCI:SCC103	24 hr; 24 hr washout	18.01 ± 2.29	2.24 ± 1.17	14.85 ± 1.67	9.12 ± 0.86
	24 hr; 48 hr washout	12.87 ± 1.51	2.48 ± 1.04	10.87 ± 0.96	9.68 ± 0.86
	24 hr; 72 hr washout	8.41 ± .095	2.53 ± 0.48	10.44 ± 1.59	7.42 ± 1.47
	24 hr; 96 hr washout	6.93 ± 1.54	1.42 ± 0.92	6.81 ± 1.17	5.15 ± 1.31
	no treatment	1.88 ± 0.90	0.61 ± 0.76	1.25 ± 0.77	1.40 ± 0.55
	24 hr; 0 washout	5.73 ± 2.37	1.93 ± 1.35	7.91 ± 1.69	2.47 ± 1.14
GM03349	24 hr; 24 hr washout	5.55 ± 1.62	1.84 ± 1.11	4.14 ± 1.32	1.71 ± 0.77
	24 hr; 48 hr washout	2.89 ± 1.59	0.45 ± 0.50	2.97 ± 1.20	1.96 ± 0.83
	24 hr; 72 hr washout	3.121 ± 1.19	1.22 ± 0.76	1.49 ± 0.95	2.27 ± 1.70
	24 hr; 96 hr washout	2.32 ± 0.97	0.94 ± 0.85	0.92 ± 0.98	2.78 ± 1.31

2