G_2/M blockade by paclitaxel induces caveolin-1 expression in A549 lung cancer cells: caveolin-1 as a marker of cytotoxicity.

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Abstract

Caveolin is a protein highly expressed in terminally differentiated cells. Caveolin-1 expression is down-regulated in various cancer cell lines. It has been shown in previous studies that exposure to low doses of paclitaxel (taxol) is sufficient to strongly up-regulate caveolin 1 and 2 protein levels. Here we show that interestingly, this up-regulation is sustained after the cessation of a paclitaxel treatment with cytostatic dose treatment and seem to be blocked into this phase. When paclitaxel is removed, there is an important cell death. We tested different conditions to establish the kinetic of this observation and to study cell viability after treatment and after recovery period. Our results suggest that Caveolin could be implicated in cell death process. Concerning post treatment cell

death, we think that in cancer treatment, paclitaxel induced cell death could arrive as much during the treatment itself as during recovery period between each treatment.

Introduction

Caveolae are plasma membrane organelles which are present in almost all cell types and are abundantly found in terminally differentiated cells like endothelial cells, fibroblasts, adipocytes and muscle cells. Caveolae act as signaling protein concentrators and thus allow cross-talk between different signaling pathways. Caveolin, a 21-24kD membrane protein is the principal component and structural protein of caveolae. Caveolin has been shown to bind with signaling molecules (G proteins, Src, Ras, epidermal growth factor receptor, protein kinase C, adenylyl cycalse, and others) that are retrieved inside the caveolae and controls their activation. Caveolin maintains these proteins in an inactivated state to limit cell proliferation and this way contributes to avoid oncogenic cell transformation. In fact, a targeted down regulation of caveolin expression is proved to cause an hyperactivation of p42/44 MAP kinase cascade (Galbiati et al embo 1998). Conversely, recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage independent growth (Engelman et al jbc 1997). Caveolin-1 expression was shown to be down regulated in several lung carcnioma cell lines (Racine et al BBRC 1999) and was identified as one of twenty-six gene-products down-regulated in breast cancer cell lines (Sager et al cold Spring harbor sym 1994).

Paclitaxel is a chemotherapeutic agent that is mainly used in the treatment of breast, ovary, esophagus and lung cancer. Paclitaxel acts by its fixation to microtubules . This fixation blocks cell cycle in G_2/M phase because paclitaxel inhibits microtubule depolymerisation during chromatides separation. Depending on the concentration, different effects could be obtained. At low doses (< 5nM) for 3-4 days, paclitaxel only slows down proliferation. At therapeutic conditions (5-200nM) there is a cell cycle blokade. Higher doses of paclitaxel induces non-cell cycle dependant death (Blagosklonny et al Int.J.Cancer 83:151-156). A previous study of our research group showed that the only exposition of A549 cells to chemotherpeutic drugs like VP-16 and doxorubicine induced an up-regulation of caveolin-1 mRNA and protein expression This up-regulation was dependant on the drugs concentrations (Belanger, Anticancer Drugs. 2003 Apr;14(4):281-7). Huang Yang et al reported an up-regulation of caveolin-1 in A549 cells treated with paclitaxel for a period of 48 hours. (Febs letters 1998)
In this study, we wanted to know how the cells recover from a 4 day treatment with paclitaxel. We wanted to evaluate proliferation of cells and caveolin-1 expression levels during the treatment and how proliferation and caveolin-1 expression evolve when paclitaxel is removed.

Material and methods

Materials.

Paclitaxel, stock concentration 7mM, was from Bristol-Myers Squibb (Montreal, Qc). Anti-caveolin-1 mouse IgG (clone 2297), anti-caveolin 1 rabbit IgG and anti-caveolin-2 mouse IgG were from Transduction Laboratories (Lexington, KY).

Cell culture.

A549 cell line used in this report were obtained from the ATCC (Manassas, VA) and cultured as suggested by the supplier.

Cell proliferation studies

Cells were cultured at a predetermined number of 125000 per well in 6 wells plate for 18 hours in Dulbecco's modified eagle medium (DMEM). After this pre-treatment period, control cells were harvested using trypsin/EDTA, counted and pelleted by centrifugation after what they were lysed in Laemli buffer (100 mM Tris, 1% SDS, 10% glycerol, 5% β-mercaptoethanol and 0,02% phenol red) at $1X10^6$ cells/mL. The other cells were incubated for a period of paclitaxel treatment in DMEM. For treatment period longer than 2 days, DMEM and paclitaxel were renewed until the end of the experiment. For certain experiments, cells were subjected to a recovery period in which they were incubated in DMEM. After the treatment cells were collected, counted and lysed. All the lysis were frozen for ulterior western blot analysis.

Western Blot analysis.

The homogenate were boiled for 5 minutes and separated by electrophoresed on 15% denaturing acrylamide gel (15% acrylamide, 383mM Tris pH 8,3, 0,1% SDS, 0,1% tetramethylethylenediamine and 2,3% ammonium persulfate). Volumes of samples loaded on gel were corrected for the number of cells. Proteins were transferred on a nitrocellulose membrane. Non-specific proteins were blocked in 5% skim milk in TBS-T

(Tris Buffered Saline with Tween : 10mM Tris, 150 mM NaCl and 0,05% Tween 20) at 4C overnight. Membrane was hybridized with primary caveolin-1 (dilution 1/5000) or caveolin-2 (dilution 1/1000) antibodies in TBS-T for 1 hour at room temperature followed by an incubation with secondary antibody (anti-rabbit for caveolin-1 and anti-mouse for caveolin –2) for 45 minutes at a 1/2000 dilution. Between each antibody incubation, membrane was washed in TBS-T. Proteins were revealed with a chemiluminescent peroxidase substrate (NEN Life Science Products, Boston MA) and exposed with ChemiImager system (Alpha Innotech Corporation, San Leandro, CA).

Immunofluorescence staining

One hundred thousand cells were cultured in LabTech slides (Nalge Nunc Internationnal, Naperville, IL) for 4 days in presence or not of paclitaxel 25nM. After the incubation period, cells were washed with PBS (Phosphate buffered saline : 130 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4) and fixed in 100% methanol at –20C for 15 minutes. Non-specific sites were blocked with 0.5% bovine serum albumin (BSA) for 15 minutes. Anti-caveolin-1mouse IgG (dilution 1/200) was incubated for 1h at room temperature in 0.5% BSA. Anti-mouse ALEXA 488 coupled secondary antibody (Molecular Probes, Leiden, Netherlands) was used at a 1/1000 dilution in PBS and incubated 30 minutes at room temperature in the dark. After each antibody incubation, cells were washed with PBS. Finally, cells were covered with mounting media (90% glycerol, 10% PBS and 0,1% p-phenylenediamine) and a microscope slide. Cells were then observed with an epifluorescence microscope (Eclipse E600, Nikon) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. Images were

captured with a Nikon camera related to the microscope. For pictures, Kodak Gold 200 ISO films were used .

Cell cycle analysis

Cells were collected, counted and pelleted by centrifugation. Cells were then washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} and fixed in 70% ethanol at -20C for 24 hours. Cells were stained in a propidium iodide solution containing 100 units/mL of DNAse-free RNAse (Sigma, Oakville, ON) and 50 µg/ml of propidium iodide (R&D Systems, Minneapolis, MN) in PBS for 30 minutes at room temperature and then incubated at 4C for at least 24 hours before flow cytometry analysis. Analysis were performed on a Coulter Elite flow cytometer (Becton Dickinson, Fullerton, CA). Ten thousand events were recorded for each sample.

Proliferation study by flow cytometry

Cells were cultured has described previously. They were incubated with and without 5-bromo 2'-deoxyuridine (BrdU) 60 µM (Sigma) for 3 hours. Cells are then harvested as described in the previous section, fixed in 70% ethanol and placed at 4C for at least 24 hours. Cells were treated with RNAse A 20U/mL for 30 minutes at 37C and incubated in HCl 2N, for 20 minutes at room temperature. Cells are then immuno-stained or not with an anti-BrdU mouse antibody conjugated with ALEXA 488 dye (Molecular Probes, Leiden, Netherlands) at 5 µg/mL in Hank's Balanced Salt Solution (HBSS) with 0,1% Triton X-100 and 0,5% BSA. The incubation is done at room temperature in the dark for 30 minutes. Cells are finally incubated with propidium iodide (PI) 50 µg/mL as described

in the previous section. For cell cycle analysis, the same parameters are studied and 10 000 events were captured. For proliferation analysis, the auto-fluorescence was discriminated from the specific fluorescence. Each individual positive staining (green staining for BrdU and red staining for PI) were determined and gated. After these adjustments, double-stained samples were analyzed to evaluate the proliferation level in a logarithmic scale in function of cell DNA content (indicator of cell cycle phase). Ten thousand events were analyzed.

3. Results and Discussion

Proliferation and expression of caveolin-1 and –2 is dose dependant in A549 cells treated with paclitaxel

To evaluate the effect of a paclitaxel treatment on cell proliferation and caveolin-1 and 2 expression in A549 cells, we submitted 125 000 cells to a 4 day treatment with increasing concentrations of paclitaxel from 0 to 200 nM. As illustrated in figure 1A, there is a paclitaxel dose-dependant cell death. We can see that cell proliferation is possible with concentrations lower than 50 nM but there is a progressive diminution of proliferation with increasing concentrations leading to the cell death observed at concentrations higher than 50 nM. We called 50 nM a cytostatic dose, which means that we had the same number of cells at the beginning and at the end of the experiment. We used this concentration all along this study because it allows us to get an interesting quantity of cells to study the mechanisms of cell death. We also studied the expression of caveolin-1 and –2 in homogenates of cells by Western Blot for different concentrations of paclitaxel. We can see in figure 1B that there is a dose-dependant up-regulation of the expression of those 2 proteins. At higher concentrations, expression of cav-1 and –2 seems to be lowered in comparison with cytostatic concentration, mainly for cav-2.

With immunofluorescence, we can visualize the expression of caveolin-1 in A549 cells treated or not with palclitaxel 50 nM for 4 days (Figure 1C). We can see on the top picture the autofluorescence of cells which is different of specific staining observed on the bottom picture.

Paclitaxel provoke cell death, caveolin-1 and –2 up-regulation and cell cycle blockade during the treatment and these modifications are amplified or maintained when paclitaxel is removed.

We wanted to determine the effect of a 4 day treatment followed by different recovery periods on proliferation, caveolin-1 expression and cell cycle distribution. 125 000 cells were submitted to 50 nM paclitaxel for 4 days. After this treatment, paclitaxel was removed and cells were kept in DMEM without drug for 1 to 4 days. Cells were collected, counted, lysed or prepared for flow cytometry for all of these conditions. We can see in figure 2A with cell counts that the 50 nM paclitaxel treatment was cytostatic as we expected. When we removed the drug we did not see a proliferation resumption. One day after the end of the treatment, about the half of cells were alive and cell death was increased proportionally with the duration of the recovery period. The analysis of caveolin-1 and -2 expression by Western Blot was done with all conditions of the experiment. We can see on figure 2B an up-regulation of caveolin-1 and –2 expression during the treatment and this up-regulation remains after the treatment. For caveolin-2 there is a second up-regulation during the recovery period. With flow cytometry analysis, we can see the progression of cells through cell cycle during treatment and for different recovery periods (figure 2B). Without treatment, standard cell distribution is respected with a majority of cells in G_1 phase with a DNA content of 2n chromosomes. With a 4 day treatment with paclitaxel 50 nM, we can see an important number of cells in G_2 / M phase, with a cell DNA content of 4n chromosomes. There is also an important number of cells in all intermediate DNA contents in this cell population, witch means that there is DNA degradation. After the end of the treatment, we can see that the same distribution of the cell population is maintained.

Paclitaxel treatment duration influence post-treatment cell death and caveolin-1 expression.

We wanted to study the effects of different periods of treatment on the recovery of cells after treatment and the relation with caveolin-1 expression for these different conditions. 125 000 cells were seeded and treated for 1,2,3 of 4 days with paclitaxel 50 nM. After the treatment, cells were incubated without drug for 3 days after what they were counted and lysed for western blot analysis. In figure 3A we can observe that during the first day of treatment, there is cell death and during the second day, cells proliferate with a diminution of proliferation during the third day until the fourth day where we observe the ultimate 4 day cytostatic effect. One day of treatment does not seem to be sufficient to induce post-treatment cell death but it reduces cell proliferation allowing only few cells to proliferate. Conversely, after 2 days of treatment, we can see a cell death when we remove drug. This post-treatment cell death is proportional to treatment duration. With western blot analysis showed in figure 3B we can see an up-regulation of caveolin-1 expression proportional to the duration of the treatment. When the drug is removed, we can see a second up-regulation of expression. The difference of expression between treatment and post-treatment is decreased proportionally with the duration of treatment.

Paclitaxel treatment allows cell survival for few cells but inhibits proliferation

We wanted to evaluate cell viability after paclitaxel treatment and recovery period. We seeded 300 000 cells and treated them with paclitaxel 50 nM for 4 days (figure 4A). Two experiments were done in parallel. For one experiment, cells were counted after 4 days and re-seeded for 3 hours or 3 days. For the other experiment, paclitaxel was removed and cells were kept in DMEM without paclitaxel for 3 days. After this post-treatment period, cells were counted and

re-seeded for 3 hours or 3 days. For both experiments, the re-seeding period was followed by cell counts and cells were lysed for western blot analysis. The results of cell counts (figure 4B) show that only half of the treated cells could stick to the plastic after 3 hours and these cells could not proliferate as we can see with the cell counts after 3 days. Cells incubated without paclitaxel for 3 days after treatment died in a large proportion as we expected. After re-seeding, only a quart of the cells could adhere to the plastic after 3 hours and they did not proliferate after 3 days. In western blot analysis (figure 4C), we can see for both experiments that treated and post-treated cells over-express caveolin-1 after a re-seeding period of 3 hours in comparison with control cells. There is a second up-regulation of caveolin-1 expression after 3 days of incubation.

The effects of paclitaxel treatment on cell viability was also studied by cell cycle analysis with flow cytometry. Cells were harvested at different times : 0, 4 days of paclitaxel 50 nM, 3 hours after re-seeding and 3 days after re-seeding. The non adhering cells were also harvested and analyzed. All these cells were fixed in ethanol and stained with propidium iodide. Results are shown in figure 5. On panel 5A, we can see a standard cell cycle distribution, with a majority of cells in G_1 phase. After 4 days of treatment (panel 5B) we can see the expected G_2 / M blockade, with an important DNA degradation. The non-adhering cells (panel 5C) seem to present an important DNA degradation with some cells concentrated in G_2 / M phase. The adhering cells after 3 hours (panel 5D) are mainly blocked in G_2 / M phase. After 3 days of incubation, the G_2 / M peak is shifted toward G_1 phase, probably due to a DNA degradation of an important number of cells.

Treated cells in S phase of cell cycle are not in DNA synthesis process.

After a treatment period, we can see some cells in S phase of cell cycle even if paclitaxel is known to inhibit cell proliferation. To determine if the S phase cells were proliferating cells or not, we decided to evaluate cell proliferation by BrdU incorporation in function of cell cycle phase. We treated cells for 0, 4 days or 4 days followed by 3 days without paclitaxel. For each condition we incubated cells with BrdU before fixing them. With a double staining with anti-BrdU antibody and propidium iodide, we analyzed by flow cytometry proliferation for cells distributed in the different phases of cell cycle. We can see on the top row cell cycle distribution, and on the bottom row results of double staining. Bottom row cell cycle phases are superimposed on those of the top row. Control cells show the normal distribution of cell cycle with a peak in G_1 phase (6A) and a positive staining of BrdU for cells in S phase (6D). Cells treated with paclitaxel for 4 days show the typical cell cycle blockade in G_2 / M phase (6B) and cells in S phase remain negative for BrdU (6E). For cells treated 4 days and kept in paclitaxel free medium for 3 additional days, the same pattern is repeated for cell cycle distribution (6C) and for evaluation of proliferation with BrdU (6E). Cells treated with paclitaxel and found in S phase are probably rather apoptotic cells than proliferating cells considering the negative staining with BrdU.

Caveolin-1 expression is known to be abolished in transformed fibroblasts (48-50 mémoire). Cells with caveolin-1 anti-sens show constitutive activation of different oncogenes (Src, Ras, v-abl) that have mitogenic properties. Expression of caveolin is important for the maintenance of a differentiated phenotype. It has also been observed by Lisanti et al that treatment with paclitaxel provoke an up-regulation of caveolin-1 expression. This observation was also done

with other drugs like doxorubicin and VP16 in our research group. These results lead us to the hypothesis that caveolin-1 could be a cytotoxicity marker(belanger). We then wanted to understand related mechanisms and we were curious to know how cells would recover after a period of treatment. We kept cells in paclitaxel free medium for 1 to 4 days and we were surprise to observe that there was an important cell death during the recovery period. An other objective followed from this observation. So the final aim of this study was to understand mechanisms of paclitaxel induced cell death and post treatment cell death and to relate this cell death with the increase of caveolin-1 expression.

The mechanism of action of paclitaxel is known. Paclitaxel enters in cells and binds to a precise site on microtubules. This fixation inhibits microtubule depolymerisation which is essential for cell division. Cells are then blocked in G2 / M phase of cell cycle and cannot proliferate 26;34;35 81. We propose the following hypothesis to explain the important cell death noticed after the treatment. When the treatment is stopped, there is no more entry of paclitaxel in cells. Then begin a degradation period during which remaining paclitaxel is eliminated (33). With no paclitaxel to inhibit microtubule depolymerisation, cells can proliferate but their chromosomal content is still abnormal. When cells try to enter G1 phase, several checkpoints like p53, Rb and cdk2 kinase are activated. These proteins prevent cell cycle progression and lead cells to apoptosis (33).

In other studies, some cells were able to escape G2 / M blockade and proliferate with a DNA content larger than 4n (83-85 dans mémoire). They used cell lines that have mutations for G1 checkpoints. We did not observe this phenomenon because A549 cells are not mutated for G1 checkpoints (84;85). Concentrations and duration of treatment could influence the ability to proliferate after treatment(33). If treatment is shorter than 48, its possible that some cells have

not passed G2 / M phase. With longer period of treatment chances of passing through G2 / M are larger (81). Furthermore, many signalization pathways are closely related to microtubules. Paclitaxel treatment could influence directly their activation state and thus slows proliferation and cell cycle progression 34;35(25).

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Figure 1

Dose dependant proliferation and expression of caveolin-1 and –2 during treatment with palcitaxel in A549 cells.

A549 cells were cultured for 4 days at increasing concentration of paclitaxel from 0 to 200 nM in 6 wells plates (A and B) or at a cytostatic concentration of 50 nM in Labtech chamber slides (C). Cells were harvested, counted and lysed (A and B). The number of cells / well in function of paclitaxel concentration is shown in panel A. Panel B presents the expression of caveolin-1 and –2 in function of paclitaxel concentration in 50 000 cells homogenised, submitted to SDS-PAGE electrophoressis and immuno-blotted with specific antibodies. Panel C shows the expression of caveolin-1 by immunofluoresence in control cells (top picture) and in paclitaxel treated cells (bottom picture)

Figure 2

Cell proliferation and caveolin-1 and –2 expression in A549 cells treated and post-treated with paclitaxel 50 nM for 4 days.

A549 cells were treated 0 or 4 days with paclitaxel 50 nM followed by a recovery period of 1,2,3 or 4 days. For each condition, cells were harvested, counted and lysed for western blot analysis. Here we can see the effects of the treatment and the stopping of the treatment on cell proliferation in A549 cells. The graph shows average cell counts (n=3, ± sem) in function of time (A). Caveolin-1 and –2 were also evaluated in function of time by Western Blot with 50 000 cells homogenates (B). Cell cycle distribution was studied by flow cytometry. Panel C presents the number of events in function of cell cycle phase and in function of time for a total number of events of 10 000.

Figure 3

Cell proliferation and caveolin-1 expression dependant on paclitaxel treatment duration in A549 cells.

A549 cells were treated for 1, 2, 3 or 4 days with paclitaxel 50 nM followed by a recovery period of 3 days. For each condition, cells were collected, counted and lysed. This graph shows the average of cells/well (n=3, ± sem) for each duration of treatment and its respective recovery period (A). Homogenates of 50 000 cells were subjected to SDS-PAGE electrophoresis and immoblotting with caveolin-1 antibody (B).

Figure 4

Stable state of A549 cells after un treatment and recovery period with paxlitaxel: cells stay alive, but cannot proliferate.

A549 cells were treated for 4 days with paclitaxel 50 nM or 4 days followed by a recovery period of 3 days. For each condition cells were collected, counted and lysed. Some cells were kept and returned to culture for 3 hours or 3 days. Panel A represents the time line of this experiment. Panel B shows average cell counts (n=3, ± sem) for each different culture conditions. Homogenates of 50 000 cells were studied by Western Blot for the expression of caveolin-1 for each of these conditions as shown in panel C.

Figure 5

Cell cycle distribution of re-adhering cells treated with paclitaxel and returned to culture.

A549 cells were treated (B) or not (A) for 4 days with paclitaxel 50 nM and re-seeded for a period of 3 hours (D) or 3 days (E). For each condition, cells were collected, counted, fixed in ethanol and stained with propidium iodide before analysis by flow cytometry. These histograms show the number of events in function of concentration of PI, which represents DNA content for each cell.

Figure 6

A549 cell proliferation through cell cycle phases during and after a treatment with paclitaxel

A549 cells were cultured for 4 days with (B and E) or without (A and D) palitaxel 50 nM followed by a three day recovery period (C and F). Cells were incubated with BrdU for 3 hours before they were collected, counted and fixed with ethanol. Cells were then double-stained with an anti-BrdU ALEXA 488 conjugated and with PI. Flow cytometry analysis were done to study cell cycle (A,B,C) and to evaluate cell proliferation through the cell cycle (D,E,F). Top histograms show number of events in function of PI concentration per cell. Bottom histograms show the concentration in BrdU (proliferation) in function of PI concentration (cell cycle phase).

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Figure legends

Figure 1 Paclitaxel induces caveolin expression in A549 cells. A) Cells were treated with increasing doses of Paclitaxel for 4 days. Two hundred thousand cells (dotted line) were plated per well on day 0 in triplicate. Cells were trypsinized and counted on a hemacytometer. Cell counts are expressed in cells/well ± standard error of the mean (SEM) (n=3). B) Volumes of crude cell homogenates corresponding to 30000 cells were separated by SDS-PAGE and Western blot analysis was made using caveolin 1 or 2 monoclonal antibodies. C) Cells were cultured in chamber slides with the indicated concentration of Paclitaxel for 48 hours. Cells were then fixed and immunofluorescence for caveolins 1 was performed as described in the Material and Methods section. Note the more abundant grainy labeling characteristic for caveolin in the treated cells.

Figure 2 Cessation of a cytostatic paclitaxel treatment induces increased cell death and a sustained expression of caveolins. A) Cells were treated as indicated then cultured for 1 to 3 days without drugs. Adhering cells at the end were counted as indicated above and results are expressed in cell/well ± SEM (n=3). The horizontal dotted line indicates the number of cells plated at day 0. The vertical line shows when the treatment has been stopped. B) Caveolin 1 and 2 protein levels increase caused by paclitaxel treatement is sustained afterwards.

Figure 3 Cessation of paclitaxel increased caveolins expression. A) Cells were treated with 50 nM of paclitaxel for up to 4 days then cultured or not for 3 days without

drug. Open bars indicate cell counts after paclitaxel whereas hatched bars indicate cell counts after the additional 3 days of culture in normal conditions. Adhering cells were counted as indicated above and results are expressed in cell/well ± SEM (n=3). The horizontal dotted line indicates the number of cells plated at day 0. B) Caveolin 1 protein levels are increased after return to normal culture conditions in A549 cells treated with paclitaxel for up to four days. C) Paclitaxel causes cell cycle blockade in the G2/M phase and this blockade is sustained after return to normal culture conditions Cell cycle analysis was realized as described in the Material and Methods section.

Figure 4 A slow loss of viability is observed after a cytostatic treatment with Paclitaxel in A549 cells. A) A549 cells were cultured for 4 days (open bars) with paclitaxel (50 nM) or for four days plus an additional three days of normal culture conditions (hatched bars) (left). Cells were then trypsinized and put back in culture for 3 hours or 3 days. Adhering cells were counted as indicated above and results are expressed in cell/well ± SEM (n=3). B) Caveolin 1 levels are increased in viable cells after paclitaxel treatment.

Figure 5 Cell cycle analysis of non-adhering and adhering cells from the experiment described in Figure 4 (paclitaxel 4 days + no drug for 3 days). A) Proliferating A549 cells. Note the small white lines indicating the G_0/G_1 (left) and the G_2/M (right) phases of the cycle. B) A549 cells after seven days (4 + 3) of treatment. Cells were let to adhere in normal culture conditions for three hours. The cell cycle of non-adhering (C)

and adhering cells (D) were then analyzed. E) Adhering cells after three additional days of culture. Note the left shift of the G_2/M peak present in D.