

DOCETAXEL INDUCES P53-DEPENDENT APOPTOSIS AND SYNERGIZES WITH FARNESYL TRANSFERASE INHIBITOR R115777 IN HUMAN EPITHELIAL CANCER CELLS

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1. ABSTRACT

Docetaxel (Taxotere, DTX) is a promoter of apoptosis in cancer cells. Since cytotoxic mechanisms of DTX are not yet fully understood, we have investigated the effects of DTX on apoptosis and ras->Erk-mediated signal transduction in human epidermoid KB, colon HT-29 and breast HCC1937 cancer cells. We have found that the exposure to 0.78 or 1.56 or 2.5 ng/ml DTX for 48 h induced apoptosis and growth inhibition in about 50 % of KB, HCC1937 and HT-29 cell population, respectively. In these experimental conditions, PARP and caspase 3 cleavage was also showed in all cell lines. KB and HCC1937 cells express a wild type p53 while HT-29 display a mutated form. Interestingly, we have found that DTX reduces the expression of mutated p53 in HT-29 and increases the expression of wild type in KB and HCC1937 cells. Moreover, DTX reduces ubiquitination of the wild type p53 in KB and HCC1937 cells and increases the ubiquitin-conjugated form of mutated p53 in HT-29 cells. Furthermore, exposure of cancer cells to DTX for 48 h increases the expression and activity of Ras and up-regulates Raf-1 and the phosphorylated isoforms of Erk-1/2. On the bases of these data, we have hypothesized that

the increased activity of the ras->erk-dependent pathway induced by DTX could be a protective signalling from the apoptosis caused by the drug. Therefore, we have used R115777, a farnesyl transferase inhibitor that inactivates ras, in combination with DTX. The combined treatment with DTX and R115777 resulted in a strong synergism in growth inhibition in the three cell lines. These data suggest the use of the combination in these therapeutic settings even if further experiments are required for the clinical translation.

2. INTRODUCTION

Docetaxel (Taxotere) is a member of the taxane class of anticancer agents to reach clinical use. This semisynthetic analog of paclitaxel (Taxol) is one of the newer potent anti-neoplastic agents now undergoing extensive laboratory and clinical investigations. Several studies indicate that antimicrotubule agents are potent promoters of apoptosis in cancer cells. Cytotoxic mechanisms of antimitotic taxoids are not yet fully understood, but it has been demonstrated that docetaxel

increases tubulin polymerisation, promotes microtubule assembly and also inhibits tubulin depolymerisation (1-3). Disruption of microtubules results also in the induction of tumor suppressor gene p53 and inhibitor of cyclin-dependent kinases and activation/inactivation of several protein kinases (1-3). As a consequence cells are arrested in the G2-M phase of the cell cycle, after which they may either undergo cell death by apoptosis or necrosis or overcome the G2-M stop and continue in the division cycle (often toward a post-mitotic cell death) depending on the tumor cell type. Nevertheless, how docetaxel induces apoptotic cell death or caspases activation is not yet defined. One may assume that taxanes are able to induce the phosphorylation of Bel-X(L)/Bel-2 members and thus inactivate their anti-apoptotic capacities. The down-regulation of Bel-2 and/or the upregulation of p53 and p21/WAF-1 are certainly one of the important modes of apoptosis induction by taxanes. (4).

Oncogenic mutations in the *ras* gene are prevalent in human cancer, including up to 50% of colon cancers and more than 90% of pancreatic carcinomas (5). In normal cells, RAS switches between an inactive GDP-bound and an active GTP-bound state which can initiate several intracellular signaling pathways (6). RAS signaling is terminated by hydrolysis of GTP to GDP in a reaction that is stimulated by guanosine triphosphatase-activating proteins. As a consequence of specific mutational events in the *ras* sequence, oncogenic Ras proteins have a greatly reduced capacity to hydrolyze GTP. This leads to constitutive activation of down-stream signalling pathways resulting in unregulated cellular proliferation (5,7). Three *ras* genes encode four ras protein isoforms (H-*ras*, N-*ras*, K-*ras4A*, and K-*ras4B*) with K-*ras4A* and K-*ras4B* being splice variants of the same gene transcript (7). Although the functional differences between the four isoforms remain unknown, oncogenic mutations of different isoforms predominate in different tumors (8). H-*ras* mutations are generally found in carcinoma of the bladder, kidney and thyroid. N-*ras* mutations are found in myeloid and lymphoid cancers, liver carcinoma and melanoma. K-*ras* mutations predominate in colon, lung and pancreatic carcinomas.

Many lines of evidence suggest that antitumor activity can be achieved by interfering with the function of oncogenic RAS proteins (9-12). Signal transduction by Ras is dependent on its plasma membrane localization. This localization is supported by a series of post-translational modifications, the first of which is farnesylation of a Cys residue near the C-terminus of RAS proteins. This reaction is catalyzed by farnesyl protein transferase (FPT). RAS prenylation is critical for proper membrane localization and function (13-15). Therefore, FPT inhibition is a potential mechanism for interfering with RAS-driven tumor growth.

Prenylation of Ras proteins is complex. In vitro, both K- and N-RAS proteins can serve as substrates for a related protein prenyl transferase, geranylgeranyl protein transferase-1 (GGPT-1) (16, 17). Although this reaction occurs with a lower catalytic efficiency than the farnesylation of these proteins, geranylgeranylation of K-

and N-RAS proteins has been observed in cells treated with FPT inhibitors (FTIs) (18, 19). In contrast, the H-RAS protein is not a substrate for GGPT-1 in vitro or in cells treated with FTIs. Despite this alternative prenylation, FTIs demonstrate in vitro and in vivo antitumor efficacy in a variety of preclinical cancer models (20-23). Therefore, the observed activity of FTIs may, in some cases, be due to the inhibition of farnesylation of proteins in addition to or other than RAS.

R115777 is an orally active, potent, and selective inhibitor of the FPT enzyme. R115777 is a competitive inhibitor of the CAAX peptide binding site of FPT with a K_i of 0.5 nM and which inhibits the farnesylation of lamin B1 and ras (24). Furthermore, R115777 inhibited the proliferation of both H-ras-transformed fibroblasts and human colon and pancreatic cell lines and showed potent oral antitumor activity against human LoVo colon and human CAPAN-2 pancreatic xenografts (14, 20, 21). An initial Phase I trial with oral R115777 (25–1300 mg twice daily for 5 days every 2 weeks) identified dose-limiting toxicities of neuropathy and fatigue with some nausea, vomiting, and headache (10). Additional trials using either continuous twice-daily oral dosing or dosing for 21 days followed by 7 days of rest, reported a dose-limiting toxicity of myelosuppression (25). The recommended dose for Phase II trials based on this schedule was 300 mg p.o. twice daily.

In the studies reported here, we examined the effects of docetaxel on the growth inhibition and apoptosis of different epithelial cancer cell lines of different origins. Moreover, we have evaluated the mechanism of apoptotic effects and the activation status of the ras->Erk-dependent pathway in Docetaxel-treated cancer cells. Finally, we have used docetaxel/R115777 in order to achieve synergistic effects on the growth inhibition of the cancer cell lines.

3. MATERIALS AND METHODS

3.1. Materials

DMEM, BSA and FBS were purchased from Flow Laboratories (Milan, Italy). Tissue culture plasticware was from Becton Dickinson (Lincoln Park, NJ). DTX was a gift of Aventis-Pharma. R115777 was a gift of Orthobiotech (Orthobiotech, Janssen Research Center, NJ). Receptor grade EGF, farnesol and protein Sepharose were purchased from Sigma (St. Louis, MO). Rabbit antisera raised against β tubulin, Erk-1 K-23 and Erk-2 MAb C-14 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum raised against PARP was purchased by Upstate Biotech. (Lake Placid, NY). Anti-Akt MAb and the relative activity evaluation kit was purchased by Cell Signalling. Anti-pan-Ras MAb clone 10 and U0126 were purchased from Calbiochem.

3.2. Cell culture

The human oropharyngeal epidermoid carcinoma KB, the colon cancer HT29, the breast cancer HCC1937 cell lines, were obtained from the American Type Tissue Culture Collection, Rockville, MD, were grown in DMEM supplemented with heat inactivated 10% FBS, 20 mM

HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% l-glutamine and 1% sodium pyruvate. The cells were grown in a humidified atmosphere of 95% air/5% CO₂ at 37 °C.

3.3. Drug combination studies

For the study of the synergism between docetaxel (DTX) and R115777 on cell growth inhibition of HT29, HCC1937 and KB, the cells were seeded in 96-multiwell plates at the density of 5 x 10³ cells/well. After 24 h incubation at 37°C the cells were treated with different concentrations of R115777 and DTX. Drug combination studies were based on concentration-effect curves generated as a plot of the fraction of unaffected (surviving) cells versus drug concentration (Chou T.C. and Talalay P., 1984) after 72 h of treatment. To explore the relative contribution of each agent to the synergism, three combinations with different R115777/DTX molar ratios were tested for each schedule: equiactive doses of the two agents (IC₅₀), higher relative doses of R115777 (IC₇₅ of R115777/ IC₂₅ of DTX) and higher relative doses of DTX (IC₂₅ of R115777/IC₇₅ of DTX). Assessment of synergy was performed quantitating drug interaction by Calcsyn computer program (Biosoft, Ferguson, MO). Combination index (CI) values of < 1, 1, and > 1 indicate synergy, additivity, and antagonism, respectively.

3.4. Western blot analysis

KB, HT29 or HCC1937 cells were grown for 48 h with or without DTX and/or R115777 at 37°C. For cell extract preparation, the cells were washed twice with ice-cold PBS/BSA, scraped, and centrifuged for 30 min at 4 °C in 1 ml of lysis buffer (1% Triton, 0.5% sodium deoxycholate, 0.1 NaCl, 1mM EDTA, pH 7.5, 10 mM Na₂HPO₄, pH 7.4, 10 mM PMSF, 25 mM benzamidin, 1 mM leupeptin, 0.025 units/ml aprotinin). Equal amounts of cell proteins were separated by SDS-PAGE. The proteins on the gels were electro-transferred to nitrocellulose and reacted with the different MABs.

3.5. Affinity precipitation of Ras

KB, HCC1937 and HT29 cells were treated with DTX as described above. The cells were lysed in the Mg²⁺ buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA and 2% glycerol. Then, 10 µl Ras Binding Domain (RBD) conjugated to agarose was added to 1 mg of cell lysate and the mixture was incubated at 4°C for 1 h. The agarose beads were collected by microcentrifugation at 14,000 x g for 5 sec. and washed three times with Mg⁺² buffer. The agarose beads were boiled for 5 min in 2X Laemmli sample buffer and collected by a microcentrifuge pulse. The supernatants were run on 12 % SDS-PAGE, then the proteins were electrotransferred on a nitrocellulose film. The nitrocellulose was incubated overnight with 1µg/ml of anti-Ras MAB, clone RAS10 and with a secondary MAB, a goat α-mouse HRP conjugated IgG, for 1,5 h. The film was washed with PBS/0,05% Tween 20 and detected by ECL, chemiluminescence's technique (Amersham).

3.6. Internucleosomal DNA fragmentation (Ladder)

For all apoptosis evaluation experiments (gel ladder and FACS analysis) both attached and suspended

cells were collected prior the processing. DNA fragmentation was measured after extraction of low molecular weight DNA. Briefly, 10 x 10⁶ cells were resuspended in 900 µl 1 X Tris-EDTA buffer and lysed with 25 µl 20% SDS. DNA was precipitated in ethanol for 6 h in the presence of 5 M NaCl. The high molecular weight fraction was sedimented by high-speed centrifugation, and the fragmented DNA was extracted from the aqueous phase with phenol and chloroform and then precipitated with ethanol. After resuspension in water, DNA was electrophoresed using 1.5% agarose gel and visualized by ultraviolet light following ethidium bromide staining.

3.7. Flow cytometric analysis of apoptosis

Apoptotic cell death was analysed by Annexin-V-FITC staining and by propidium iodide (PI) detection systems. Annexin-V-FITC binds to phosphatidylserine residues, which are translocated from the inner to the outer leaflet of the plasma membrane during the early stages of apoptosis. Labelling of apoptotic cells was performed using an Annexin-V kit (MedSystems Diagnostics, Vienna, Austria). Briefly, cells were incubated with Annexin-V-FITC in a binding buffer (provided by the manufacturer) for 10 min at room temperature, washed and resuspended in the same buffer as described by the manufacturer. Analysis of apoptotic cells was performed by flow cytometry (FACScan, Becton Dickinson). Propidium iodide analysis of apoptosis was performed using a commercial kit (MedSystems Diagnostics, Vienna, Austria). The cells were washed in PBS, resuspended in 190 µl of prediluted binding buffer (1 : 4) and incubated for 10 min with 10 µl of the 20 µg/ml propidium iodide stock solution, and then the apoptotic cells were analysed by FACScan flow cytometer. For each sample, 2 x 10⁴ events were acquired. Analysis was carried out by triplicate determination on at least three separate experiments.

3.8. Fluorescence Microscopy

After washing in PBS, cells were treated with in situ detection kit, according to manufacturers (SantaCruz Biotechnology CA). In details, cells were incubated with PI and a FITC-conjugated antibody raised against annexin V for at 37 °C at the dark. Then, cells were observed under fluorescent microscope using a dual filter set for FITC and rhodamine. The images were acquired with a dedicated software.

3.9. Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed by analysis of variance (ANOVA) with Neumann-Keul's multiple comparison test or Kolmogorov-Smirnov where appropriate.

4. RESULTS

4.1. Human epithelial cancer cells are differently sensitive to DTX

We have evaluated the antiproliferative effects of DTX on three different cell lines: one derived from human epidermoid cancer (KB) with wild type p53 and other two derived from colon cancer (HT29) and breast

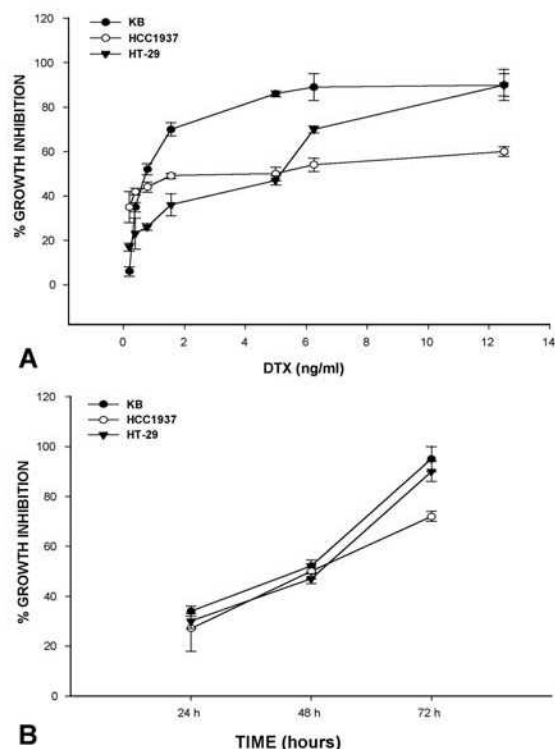


Figure 1. DTX induces time, dose and p53-dependent growth inhibition in human epithelial cancer cells. KB, HCC1937 and HT-29 cells were seeded and treated with DTX at different concentrations (A) and exposure times (B) as described in Materials and Methods. (A) % growth inhibition induced by different concentrations of DTX after 48 h of treatment in human epidermoid KB (●), colon HT-29 (▼) and breast HCC1937 (○) cancer cells evaluated with MTT assay. Each point is the mean of at least four different replicate experiments. Bars, SEs. (B) Human epidermoid KB (●), colon HT-29 (▼) and breast HCC1937 (○) cancer cells were seeded and treated with 0.78 ng/ml DTX (for KB cells) or 6 ng/ml (for both HCC1937 and HT-29 cells) for 24, 48 and 72 h as described in "Materials and Methods". The effects on proliferation are expressed as % of growth inhibition as compared to untreated cells. Each point is the mean of at least four different replicate experiments. Bars, SEs.

adenocarcinoma (HCC1937) with mutated p53. DTX induced a time and dose-dependent growth inhibition in all three cell lines (Figure 1A and B). The 50% growth inhibition was reached at DTX concentrations of about 0.78 ng/ml for KB cells and of 5 ng/ml for both HCC1937 and HT29 cell lines after 48h of treatment with the drug (Figure 1A and B). These data suggest that the three cell lines are differently sensitive to the growth inhibition induced by DTX and the sensitivity seems to be dependent from p53 status being wild type p53 tumour cells more responsive to the treatment with the drug.

4.2. DTX causes apoptosis in human epithelial cancer cells

In these experimental conditions apoptosis was detected in all three cell lines. In fact, 0.78 ng/ml DTX

induced a time-dependent effect on apoptotic onset in KB cells as evaluated by double labelling with PI and FITC annexin V at fluorescence microscopy. Apoptosis was recorded already after 12 h exposure to the drug, but became maximal after 48 h (Figure 2 A). Similar results were found with the analysis of endonucleosomal DNA fragmentation after 48 h exposure to 0.78 ng/ml DTX for KB cells and 5 ng/ml DTX for HCC1937 and HT-29 cells (Figure 2B). In these experimental conditions about 50% of cell population was apoptotic as determined with FACS analysis after labelling with PI (Figure 2C). These data suggest that the growth inhibition induced by DTX in these cell lines was largely dependent from apoptotic effects.

4.3. DTX induces caspase activation and modulates p53 expression and ubiquitination in human cancer cells

In order to characterize the apoptosis induced by DTX in these epithelial cancer cell lines we have evaluated the effects of DTX on caspase 3 and PARP cleavage. We have found that 48 h treatment of KB cells with 0.78 ng/ml DTX and of HCC1937 and HT-29 cells with 5 ng/ml DTX induced a clear fragmentation of both caspase 3 and of its substrate PARP (Figure 3A and B, respectively). These data suggest that the apoptosis caused by DTX is likely mediated by the activation of a caspase cascade that has caspase 3 as final executioner. Since p53 is a key mediator of apoptosis in human cancer cells and the three cancer cell lines display differential mutated/wild type status (that correlated with sensitivity to DTX) we have evaluated the effects of DTX on p53 expression on all cell lines. We have found that 48 h 0.78 ng/ml DTX induced an about 3-fold increase of p53 expression in KB cells that have a wild type form of p53 (Figure 3C). On the other hand, 5 ng/ml 48 h DTX induced a 2-3-fold decrease of p53 expression on both HCC1937 and HT-29 cell lines that have a mutated p53 form (Figure 3C). These effects were paralleled by changes in p53 ubiquitination as demonstrated by western blotting with ubiquitin after p53 immunoprecipitation. In fact, we have found that, in the same experimental conditions, DTX induced a decrease of p53 ubiquitinated form in KB cells while an increase was observed in the other two cell lines (Figure 3D). These data suggest that DTX can induce modulation of p53 expression through changes in its ubiquitinated status and likely of its proteasome-dependent degradation.

4.4. DTX induces ras and Erk activation

Tumour cells activate specific survival pathways in order to protect themselves from apoptosis induced by physical or chemical agents. The ras->Erk-dependent pathway is a well known survival pathway of human epithelial cells. On the basis of these considerations we have evaluated the effects of DTX on ras and erk-1/2 activation status in the three cell lines. We have found that 48 h 0.78 ng/ml DTX in KB cells and 5 ng/ml DTX in both HT-29 and HCC1937 cells induced an about 2-3-fold increase of both ras and Erk activities (Figure 3 F and H, respectively) while no change in ras and Erk expressions was recorded (Figure 3 E and G, respectively). On the basis of these results, it can be supposed that a ras->Erk-dependent survival pathway is activated in apoptotic cells exposed to DTX.

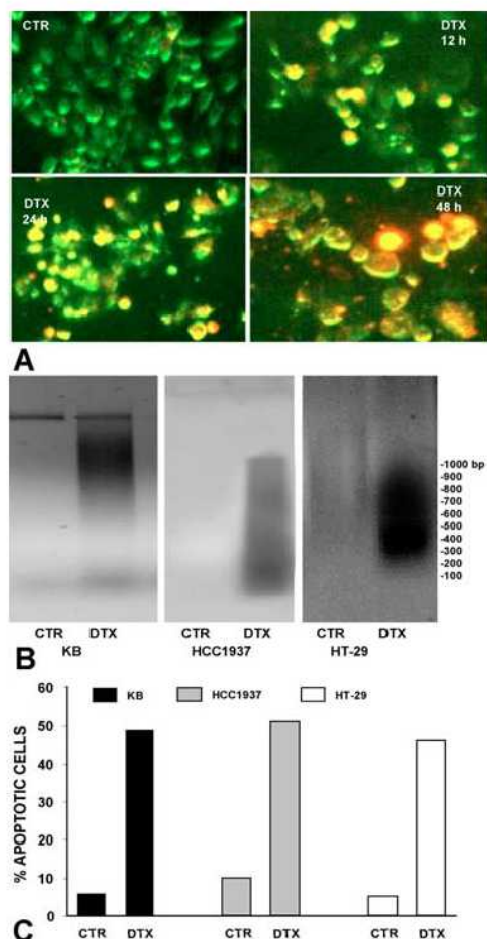


Figure 2. Apoptotic effects of DTX on human epithelial cancer cells. (A) KB cells were seeded and treated with 0.78 ng/ml DTX for different times. At the time of the experiment cells were fixed and processed at fluorescence microscopy after PI and anti-annexin V antibody labelling as described in “Materials and Methods”. The experiments were performed at least three different times and the results were always similar. Double labelled cells (yellow) are apoptotic cells. CTR, Control cells; DTX 12 h, 12 h 0.78 ng/ml DTX-treated KB cells; DTX 24 h, 24 h 0.78 ng/ml DTX-treated KB cells; DTX 48 h, 48 h 0.78 ng/ml DTX-treated KB cells. (B) The internucleosomal DNA fragmentation was assessed as described in “Materials and Methods”. The exposure of KB cells to 0.78 ng/ml DTX or of both HCC1937 and HT-29 to 6 ng/ml DTX for 48 h induced apoptosis. The experiments were performed at least three times and the results were always similar. CTR, untreated; DTX, 48 h DTX-treated cancer cells. (C) FACS analysis after PI labelling of KB cells treated with 0.78 ng/ml DTX and of HCC1937 and HT-29 cancer cells treated with 6 ng/ml for 48 h. CTR, Control cells; DTX, DTX-treated cancer cells. The experiments were performed at least three times and the results were always similar.

4.5. FTI R115777 synergizes with DTX in inducing growth inhibition on human cancer cells

On the basis of the obtained data, we have hypothesized that the specific inhibition of the ras->Erk-

dependent survival pathway activated in DTX-treated cells with the FTI and ras inhibitor R115777 could potentiate the anti-tumour activity of DTX. We have first evaluated the effects of R115777 on growth inhibition of the three tumour cell lines. We have found that FTI induced a dose and time-dependent growth inhibition in all three cell lines (Figure 4A and data not shown). The 50% growth inhibition was reached at R115777 concentrations of about 8 μM for KB, of 11 μM for HT29 and of 20 μM for HCC1937 cell lines after 48h of treatment with the drug (Figure 4A). These data suggest that the growth inhibition induced by R115777 was different in the three cell lines being HCC1937 more resistant. Thereafter, we have combined the FTI R115777 with DTX. In details, we have evaluated the growth inhibition induced by different concentrations of DTX in combination with R115777 at 72 h on KB, HCC1937 and HT-29 cells. We have performed these experiments with MTT assay and the resulting data were elaborated with the dedicated software Calcsyn (by Chou and Talalay, see also “Materials and Methods”). With this mathematical model synergistic conditions occur when the combination index (CI) is below 1.0. When CI is less than 0.5 the combination is highly synergistic and we have chosen as cut-off for a real synergistic effect CI values below 0.8. We have found that the combination of DTX and R115777 was highly synergistic when the two drugs were used at equitoxic concentrations on KB and HCC1937 cells while overwhelming concentrations of DTX were required to reproduce synergistic conditions on HT-29 (Figure 4 B-D and table 1). The best synergistic effect on KB cell growth inhibition could be achieved when DTX was added to the cells 24 h prior R115777 while, at these experimental conditions, only an additive effect was recorded in both HCC1937 and HT29 cells (Figure 4F). On the other hand, concomitant administration of DTX and R115777 was required to obtain synergistic effects on both HCC1937 and HT-29 cells while antagonizing effects were recorded on KB cells (Figure 4E). The exposure of the three cell lines to R115777 24 h prior DTX resulted antagonistic or additive (Figure 4G). In synergistic drug combinations the CI_{50%} (the combination index calculated for 50% cell survival by isobologram analysis) were 0.25 for both KB and HCC1937 and 0.35 for HT-29 cells (table 1). Therefore, the combined use of the two agents was highly synergistic on the growth inhibition of all cell lines. Dose reduction index₅₀ (DRI₅₀) represents the magnitude of dose reduction obtained for the 50% growth inhibitory effect in combination setting as compared to each drug alone. In our experimental conditions the DRI₅₀ of DTX and FTI were equal to 29 and 57 for KB, 85 and 4 for HCC1937 and 44 and 138 for HT-29 cells, respectively (table 1). These results demonstrate that a strong or a very strong synergism can be recorded on cell proliferation when the two drugs are used in combination. Effective concentrations in the combinatory experiments are possible to be reached *in vivo*.

5. DISCUSSION

DTX is a member of the taxane class of anticancer agents to reach clinical use. This semisynthetic analog of paclitaxel (Taxol) is one of the newer potent anti-

Table 1. Pharmacological interaction between DTX and R115777 on the growth inhibition of epithelial cancer cell lines

Cell lines	DTX ED50 (ng/ml)	R115777 ED50 (μ M)	Combination ratio DTX/R115777 CI50 ¹	DRIS0 ²	Interpretation
KB DTX->FTI	0.144	0.231	6.25:10 (50%vs50%)	0.25	DTX:29 FTI: 57 Very Strong synergism
HCC1937 DTX+FTI	0.025	0.33	0.78:10 (50%vs50%)	0.25	DTX:85 FTI: 4 Very Strong synergism
HT-29 DTX+FTI	0.049	0.0098	12.5:2.5 (75%vs 25%)	0.31	DTX:44 FTI:138 Very Strong synergism

¹CI₅₀ were calculated for 50% cell survival (ED₅₀) by isobologram analyses performed with CalcuSyn software., ²DRI represents the order of magnitude (fold) of dose reduction obtained for ED₅₀ effect in combination setting as compared to each drug alone.

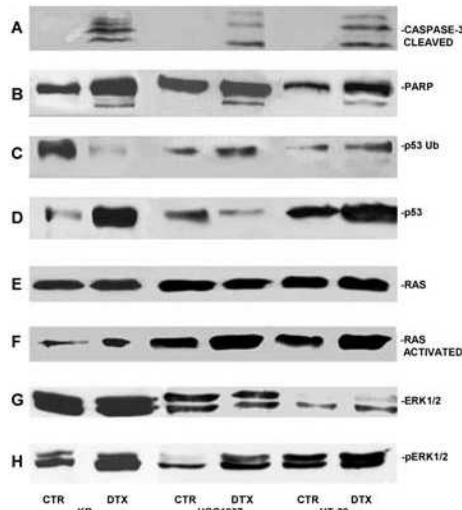


Figure 3. DTX effects on caspase 3 and PARP cleavage, p53 expression and ras->Erk-dependent pathway. The cells have been cultured for 48 h in the absence or presence of 0.78 ng/ml DTX (for KB) or of 6 ng/ml DTX (for both HCC1937 and HT-29). Then the cells were processed for the determination of both the intact and the cleaved isoforms of caspase 3 (A) and PARP (B). Moreover, the cell lysates were immunoprecipitated with a Mab raised against p53 and blotted with either an anti-ubiquitin antiserum (C) or an anti-p53 Mab (D), in order to evaluate p53 ubiquitination or expression, respectively. (E) Western blot assay for the expression of the total ras protein. (F) Affinity precipitation of ras performed with the minimal binding domain of raf-1 conjugated with agarose for the evaluation of ras activity as described in "Materials and Methods". Expression (G) and phosphorylation (H) of Erk-1 and 2 evaluated after blotting with either an anti-MAPK or an anti-pMAPK specific Mab, respectively, as described in "Materials and Methods". The experiments were performed at least three different times and the results were always similar. CTR, untreated cancer cells; DTX, 48 h DTX-treated cancer cells.

neoplastic agents now undergoing extensive laboratory and clinical investigations (4). Several studies indicate that antimicrotubule agents are potent promoters of apoptosis in cancer cells. Cytotoxic mechanisms of antimitotic taxoids are not yet fully understood, but it has been demonstrated that DTX increases tubulin polymerisation, promotes microtubule assembly and also inhibits tubulin depolymerisation (1-3). Disruption of microtubules results also in the induction of tumor suppressor gene p53 and inhibitor of cyclin-dependent kinases and activation/inactivation of several protein kinases (1-3). As a

consequence cells are arrested in the G2-M phase of the cell cycle, after which they may either undergo cell death by apoptosis or necrosis or overcome the G2-M stop and continue in the division cycle (often toward a post-mitotic cell death) depending on the tumor cell type. Nevertheless, how docetaxel induces apoptotic cell death or caspase activation is not yet defined. One may assume that taxanes are able to induce the phosphorylation of Bcl-X_L/Bcl-2 members and thus inactivate their anti-apoptotic capacities. The down-regulation of Bcl-2 and/or the upregulation of p53 and p21/WAF-1 are certainly one of the important modes of apoptosis induction by taxanes.

We have found that the epithelial tumour cell lines used as our experimental system were differentially sensitive to the growth inhibition induced by DTX and the inhibition of proliferation induced by DTX was largely due to apoptosis onset. In these experimental conditions, we have also found caspase 3 activation and the cleavage of its substrate PARP suggesting the involvement of caspase cascade in execution phase of apoptosis induced by DTX. These results are in agree with those reported from Kolfchoten et al who described that DTX induces apoptosis and caspase-3 activation occurring only in cells with accumulation in the G2/M phase starting as early as 8 hr in ovarian cancer OVCAR-3 cells (26). The same authors found also Bcl-2 phosphorylation, but they do not exclude also a caspase-3-independent cell death mechanism (26).

We have found that the wild type status of p53 correlates with the sensitivity to the growth-inhibiting effects induced by DTX. Moreover, DTX induced an increase of wild type p53 expression in KB cells that was likely due to a decrease of its ubiquitination and the consequent reduction of its proteasome-dependent degradation. On the other hand, it was recently reported that the dynamics of microtubules of mut-p53 cells were altered in complex ways and were significantly increased as compared with microtubules in wt-p53 cells as evaluated by high-resolution time-lapse fluorescence microscopy in living cells (27). In fact, the percentage of time microtubules spent in growing and shortening phases and their catastrophe frequency significantly increased in mut-p53 cells. On the basis of these findings, p53 protein may contribute to the regulation of microtubule composition and function, and alterations in p53 function may generate complex microtubule-associated mechanisms of resistance to tubulin-binding agents. Mutations in the BRCA1 gene often occur in breast and ovarian cancer and mutations that disable p53 are frequently found in human cancers, often accompanied by mutations in additional genes, contributing to tumor progression or high-grade malignancy. In fact,

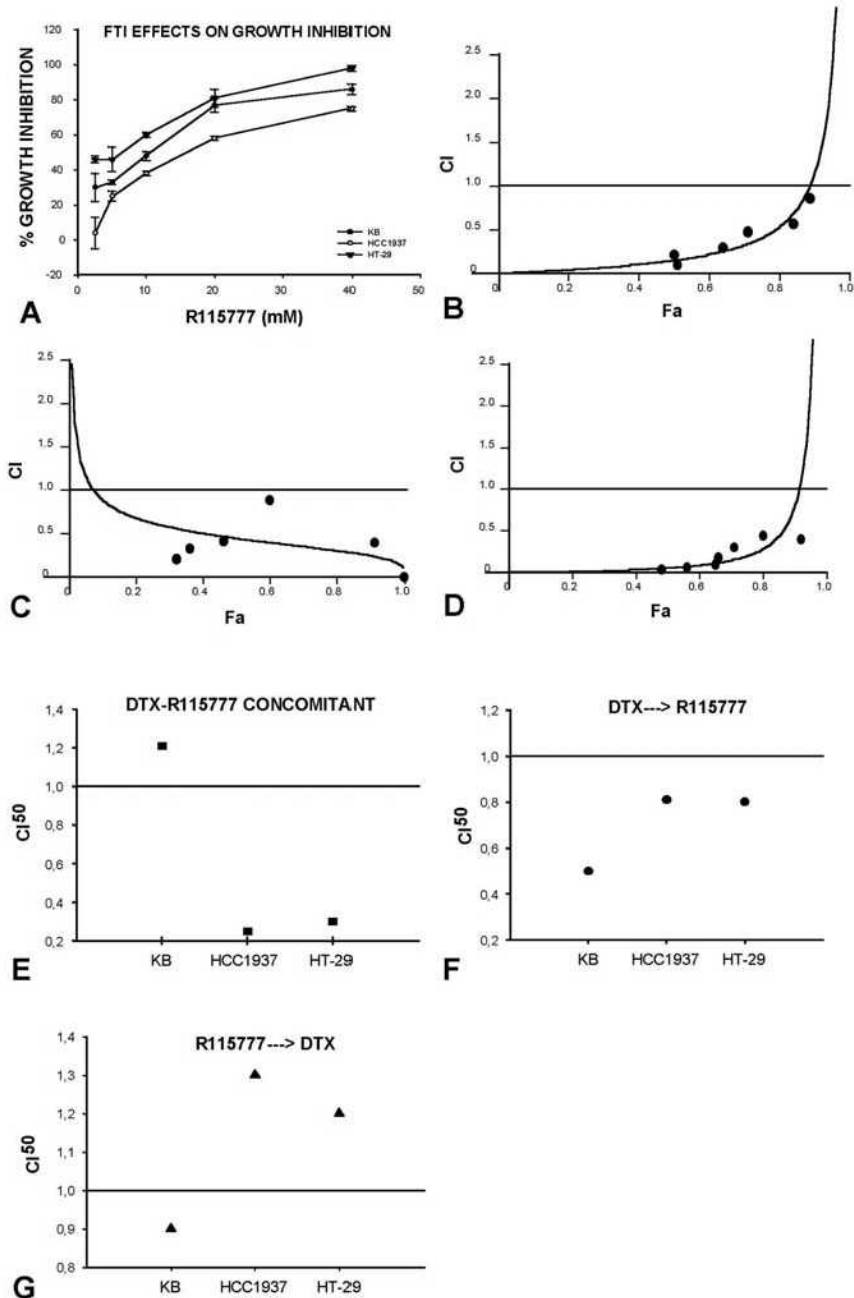


Figure 4. Pharmacological interaction between DTX and R115777 on epithelial cancer cell proliferation. (A) KB, HCC1937 and HT-29 cells were seeded and treated with FTI R115777 at different concentrations for 48 h as described in “Materials and Methods”. % growth inhibition induced by different concentrations of R115777 in human epidermoid KB (●), colon HT-29 (▼) and breast HCC1937 (○) cancer cells evaluated with MTT assay. Each point is the mean of at least four different replicate experiments. Bars, SEs. (B-D) We have evaluated the growth inhibition induced by different combinations of DTX and R115777 at 72 h on KB, HCC1937 and HT-29 cells. We have performed these experiments with MTT assay and the resulting data were elaborated with the dedicated software CalcuSyn (by Chou and Talalay) as described in “Materials and Methods”. In the Figure it is shown the isobologram analysis of the effects of DTX and R115777 combinations, used at equitoxic concentrations, on growth inhibition of KB (B, in concomitant administration setting) and HCC1937 (C, in DTX->R115777 administration setting) and of the effects of DTX and R115777 combinations, used at overwhelming concentrations of DTX, on growth inhibition of HT-29 (D, in concomitant administration setting). CI, combination index. Each point is the mean of at least four different replicate experiments. (E-G) Values of the mean CI₅₀s derived from experiments performed with the different modalities of addition of the two agents to the cells. (E) Concomitant administration of DTX and R115777; (F) DTX added to the cells 24 h prior R115777; (G) R115777 added to the cells 24 h prior DTX. CI₅₀ were calculated for 50% cell survival (ED₅₀) by isobologram analyses performed with CalcuSyn software.

HCC1937 adenocarcinoma cells lack BRCA1 expression and have a mutated p53. It was demonstrated that BRCA1-deficiency in p53-null cells was associated with decreased sensitivity to both DTX and paclitaxel (28). In our experimental conditions we have indeed found that HCC1937 cells were resistant to DTX treatment. Finally, it was also reported that the absence of p53 expression (that correlates with wild type status) in specimens derived from breast adenocarcinomas is associated with increased sensitivity to DTX (29).

On the other hand in two other studies, the in vitro sensitivity to DTX of cancer cells derived from biopsy collected from patients affected by breast tumours was not statistically correlated to p53 expressions (30, 31). Moreover, a clinical study failed in finding any correlation between p53 expression in breast cancers and response to treatment with DTX (32). On the basis of these findings, we think that conclusive remarks on p53 status and sensitivity to DTX can not be derived and that this argument requires further investigations.

Disruption of microtubule structure by antimicrotubule drugs results not only in induction of tumor suppressor gene p53, but also in activation/inactivation of several protein kinases including Ras/Raf, PKC/PKA I/II, MAP kinases, and p34cdc2 (33). In our experimental model, we have found that DTX induces an increase of the activation of both ras and Erk-1 and 2 without changes of the expression of the molecules. These results are concordant with a previous report on DTX-induced increase of raf-1 and Erk activities in human leukemic cell lines (34). On the other hand, another group described a differential effect of the two antimicrotubule drugs vinorelbine and paclitaxel on Erk 2 activity in breast adenocarcinoma MCF-7 cells. In fact, a 3-fold increase of Erk 2 activity was observed within 30 min when MCF-7 cells were treated with 0.1 μ M vinorelbine. In contrast, the same treatment with paclitaxel resulted in a significant decrease of Erk 2 activity (35). The differential effects induced by taxanes can be however dependent from both the dosages and the experimental models used.

Our data suggest that DTX can induce the hyperactivation of a ras->Erk-dependent survival pathway in epithelial cancer cells. Therefore, we have hypothesized that the selective inhibition of this pathway through the use of the FTI and ras inactivator R115777 could increase the anti-tumour activity of DTX. We have indeed found that the combination between DTX and R115777 was able to induce a synergistic effect on the growth inhibition of all the three cell lines without dependency from the p53 status. Moreover, we have demonstrated that the sequence of administration used is an important determinant of the synergistic effect and varies in the different epithelial cell lines. The combination between DTX and agents interfering with the ras->Erk-dependent pathway, such as the Mek-1 inhibitors U0126 and PD098059, has been already used by several authors. In fact, sequential, but not concomitant, treatment of MDA-MB-231 and MCF-7 cells with DTX followed by Mek 1/2 inhibitor enhanced the anti-proliferative effects of the taxane in MTT assays. (36).

Moreover, in androgen-independent C-81 LNCaP cells, inhibition of Erk by PD98059 plus DTX resulted in enhanced growth suppression and increased apoptosis, which was in part due to the inactivation of Bcl-2 by increasing phosphorylated Bcl-2 and Bax expression (37). In our experimental conditions, the best synergistic effect on KB cell growth inhibition could be achieved when DTX was added to the cells 24 h prior R115777. On the other hand, concomitant administration of DTX and R115777 was required for both HCC1937 and HT-29 cells. Moreover, equitoxic concentrations of the two drugs were needed to obtain synergistic conditions in both KB and HCC1937 cells while overwhelming concentrations of DTX were required in HT-29 cells.

In conclusion, our data suggest that DTX induces caspase 3 and p53-dependent apoptosis in human epithelial tumour cells. Moreover, an increased ras and Erk activity was recorded in tumour cells exposed to cytostatic concentrations of DTX suggesting the onset of a homeostatic anti-apoptotic pathway in DTX-treated cancer cells. The inhibition of this survival pathway induced by the FTI R115777 indeed enhanced the anti-tumour activity of DTX. These data encourage the investigation on the combined use of DTX and FTI in the treatment of human neoplasms.

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7. REFERENCES

1. Horwitz SB: Mechanism of action of Taxol. *Trends Pharmacol Sci* 13, 134 (1992)
2. Lavelle F, M. C. Bissery, C. Combeau, J. F. Riou, P. Vignaud & S. Andre: Preclinical evaluation of docetaxel (Taxotere). *Semin Oncol* 22 (Suppl 4), 3 (1995)
3. Rowinsky E. K, L. A. Cazenave & R. C. Donehower: Taxol: a novel investigational antimicrotubule agent. *J Natl Cancer Inst* 82, 1247 (1990)
4. Ganansia-Leymarie V, P. Bischoff, J. P. Bergerat & V. Holl: Signal transduction pathways of taxanes-induced apoptosis. *Curr Med Chem Anti-Canc Agents* Jul, 3 (4), 291-306 (2003)
5. Barbacid M: Ras genes. *Annu Rev Biochem* 56, 779 (1987)
6. Joneson T, M. A. White, M. H. Wigler & D. Bar-Sagi: Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. *Science* 271, 810 (1996)
7. Lowry D. R. & B. M. Willumsen: Function and regulation of Ras. *Annu Rev Biochem* 72, 851 (1993)

8. Bos J. L.: Ras oncogenes in human cancer: a review. *Cancer Res* 49, 4682 (1989)
9. Gibbs J. B., A. Oliff & N. E. Kohl: Farnesyl transferase inhibitors: Ras research yield a potential cancer therapeutic. *Cell* 77, 175 (1994)
10. Gibbs J. B., N. E. Kohl, K. S. Koblan, C. A. Omer, L. Sepp-Lorenzino, N. Rosen, N. J. Anthony, M. W. Conner, S. J. deSolms, T. N. Williams, S. L. Graham, G. D. Hartman & A. Oliff: Farnesyl transferase inhibitors and anti-Ras therapy. *Breast Cancer Res Treat* 38, 75 (1996)
11. Platter R, M. J. Anderson, K. Y. Sato, C. L. Fashing, C. J. Der & E. J. Stanbridge: Loss of oncogenic Ras expression does not correlate with loss of tumorigenicity in human cells. *Proc Natl Acad Sci USA* 93, 6665 (1996)
12. Shirasawa S, M. Furuse, N. Yokoyama & T. Sasazuki: Altered growth of human colon cancer cell lines disrupted at activated Ki ras. *Science* 260, 85 (1993)
13. Jackson J. H, C. G. Cochrane, Jr. Bourne, P. A. Soliski, J. E. Buss & C. J. Der: Farnesol modification of Kirsten-ras exon 4B protein is essential for transformation. *Proc Natl Acad Sci USA* 87, 3042 (1990)
14. Kato K, A. D. Cox, M. M. Hisaka, S. M. Graham, J. E. Buss & C. J. Der: Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. *Proc Natl Acad Sci USA* 89, 6403 (1992)
15. Schaefer W. R, R. Kim, R. Sterne, J. Thorner, S. H. Kim & J. Rine: Genetic and pharmacological suppression of oncogenic mutations in RAS genes of yeast and human. *Science* 245, 379 (1989)
16. James G. L, J. L. Goldestein & M. S. Brown Polylysine: CVIM sequences of K-RasB dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic in vitro. *J Biol Chem* 270, 6221 (1995)
17. Zhang F, R. Bond, L. Wang, W. Windsor, P. Kirschmeier, D. Carr & W. R. Bishop: Characterization of H, K- and N-Ras as in vitro substrates for isoprenyl protein transferases. *J Biol Chem* 272, 10232 (1997)
18. Rowell C. A, J. J. Kowalczyk, M. D. Lewis & A. M. Garcia: Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo. *J Biol Chem* 272, 14093 (1997)
19. Whyte D. B, P. Kirschmeier, T. N. Hockenberry, I. Nunez-Oliva, L. James, J. J. Cantino, W. R. Bishop & J. K. Pai: K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. *J Biol Chem* 272, 14459 (1997)
20. Kohl N. E, F. R. Wilson, S. D. Mosser, E. Giuliani, S. J. DeSolms, M. W. Conner, N. J. Anthony, W. J. Holtz, R. P. Gomez, T. J. Lee, R. L. Smith, S. L. Graham, G. D. Hartman, J. B. Gibbs & A. Oliff: Protein farnesyltransferase inhibitors block the growth of ras-dependent tumors in nude mice. *Proc Natl Acad Sci USA* 91, 9141 (1994)
21. Kohl N. E., C. A. Omer, M. W. Conner, N. J. Anthony, J. P. Davide, S. J. DeSolms, E. Giuliani, R. P. Gomez, S. L. Graham, K. Hamilton, L. K. Handt, G. D. Hartman, K. S. Koblan, A. M. Kral, P. J. Miller, D. Mosser, T. J. O'Neill, E. Rands, M. D. Schaber, J. B. Gibbs & A. Oliff: Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. *Nat Med* 1, 792 (1995)
22. Liu M, M. S. Bryant, J. Chen, S. Lee, B. Yaremko, Z. Li, J. Dell, P. Lipari, M. Malkowski, N. N. Prioli, R. R. Rossman, W. A. Korfmacher, A. A. Nomeir, C. D. Lin, A. K. Mallams, P. Kirshmeier, R. J. Doll, J. J. Catino, V. M. Girijavallabhan & W. R. Bishop: Effect of SCH59228 an orally bioavailable farnesyl protein transferase inhibitor, on the growth of oncogene-transformed fibroblasts and human colon carcinoma xenograft in nude mice. *Cancer Chemother Pharmacol* 43, 50(1999)
23. Nagasu T, K. Yoshimatsu, C. Rowell, M. D. Lewis & A. M. Garcia: Inhibition of human tumor xenograft growth by treatment with the farnesyl transferase inhibitor B956. *Cancer Res* 55, 5310 (1995)
24. Schellens J H, G. de Klerk, M. Swart, P. A. Palmer, C. J. Bol, L. J. van't Veer, S. Tan, G. C. de Gast, J. H. Beijnen & ten W. W. Bokkel Huinink: Phase I and pharmacologic study with the novel farnesyltransferase inhibitor (FTI) R115777. *Proc Am Soc Clin Oncol* 19, 184 (2000).
25. Zujewski, J, I. D. Horak, C. J. Bol, R. Woestenborghs, C. Bowden, D. W. End, V. K. Piotrovsky, C. R. T. Belly, A. Todd, W. C. Kopp, D. R. Kohler, C. Chow, M. Noone, F. T. Hakim, G. Larkin, R. E. Gress, R. B. Nussenblatt, A. B. Kremer, & K. H. Cowan: Phase I and pharmacokinetic study of farnesyl protein transferase inhibitor R115777 in advanced cancer. *J Clin Oncol*, 18, 927, (2000)
26. Kolfshoten G. M, T. M. Hulscher, M. C. Duyndam, H. M. Pinedo & E. Boven: Variation in the kinetics of caspase-3 activation, Bcl-2 phosphorylation and apoptotic morphology in unselected human ovarian cancer cell lines as a response to docetaxel. *Biochem Pharmacol.* 63 (4), 733-43 (2002)
27. Galmarini C. M, K. Kamath, A. Vanier-Viomery, V. Hervieu, E. Peiller, N. Falette, A. Puisieux, M. Ann Jordan & C. Dumontet: Drug resistance associated with loss of p53 involves extensive alterations in microtubule composition and dynamics. *Br J Cancer* 88 (11), 1793-9 (2003)
28. Fedier A, R. A. Steiner, V. A. Schwarz, L. Lenherr, U. Haller & D. Fink: The effect of loss of Brcal on the sensitivity to anticancer agents in p53-deficient cells. *Int J Oncol.* 22 (5), 1169-73 (2003)
29. Schmidt M, A. Bachhuber, A. Victor, E. Steiner, M. Mahlke, H. A. Lehr, H. Pilch, W. Weikel & P. G.

Knapstein: p53 expression and resistance against paclitaxel in patients with metastatic breast cancer. *J Cancer Res Clin Oncol.* 129 (5), 295-302 (2003)

30. Takamura Y, H. Kobayashi, T. Taguchi, K. Motomura, H. Inaji & S. Noguchi: Prediction of chemotherapeutic response by collagen gel droplet embedded culture-drug sensitivity test in human breast cancers. *Int J Cancer* 98 (3), 450-5 (2002)

31. Rein D. T, T. Schondorf, M. Breidenbach, M. M. Janat, A. Weikelt, U. J. Gohring, M. Becker, P. Mallmann & C. M. Kurbacher: Lack of correlation between P53 expression, BCL-2 expression, apoptosis and ex vivo chemosensitivity in advanced human breast cancer. *Anticancer Res.* 20 (6D), 5069-72 (2000)

32. Sjostrom J, C. Blomqvist, P. Heikkila, K. V. Boguslawski, A. Raisanen-Sokolowski, N. O. Bengtsson, I. Mjaaland, P. Malmstrom, B. Ostenstadt, J. Bergh, E. Wist, V. Valvere & E. Saksela: Predictive value of p53, mdm-2, p21, and mib-1 for chemotherapy response in advanced breast cancer. *Clin Cancer Res.* 6 (8), 3103-10 (2000)

33. Wang L. G, X. M. Liu, W. Kreis & D. R. Budman: The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. *Cancer Chemother Pharmacol.* 44 (5), 355-61 (1999)

34. Maestre N, C. Bezombes, I. Plo, T. Levade, F. Lavelle, G. Laurent & J. P. Jaffrezou: Phosphatidylcholine-derived phosphatidic acid and diacylglycerol are involved in the signaling pathways activated by docetaxel. *J Exp Ther Oncol.* 3 (1), 36-46 (2003)

35. Liu X. M, L. G. Wang, W. Kreis, D. R. Budman & L. M. Adams: Differential effect of vinorelbine versus paclitaxel on ERK2 kinase activity during apoptosis in MCF-7 cells. *Br J Cancer.* 85 (9), 1403-11 (2001)

36. Yacoub A, S. I. Han, R. Caron, D. Gilfor, S. Mooberry, S. Grant & P. Dent. Sequence dependent exposure of mammary carcinoma cells to Taxotere and the MEK1/2 inhibitor U0126 causes enhanced cell killing in vitro. *Cancer Biol Ther.* 2 (6), 670-6 (2003)

37. Zelivianski S, M. Spellman, M. Kellerman, V. Kakitelashvilli, X. W. Zhou, E. Lugo, M. S. Lee, R. Taylor, T. L. Davis, R. Hauke, M. F. Lin: ERK inhibitor PD98059 enhances docetaxel-induced apoptosis of androgen-independent human prostate cancer cells. *Int J Cancer.* 107 (3), 478-85 (2003)

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