Chapter - 10

In-vitro & In-vivo Characterization of Docetaxel & Its Impurities

10.1. Introduction

Docetaxel (trademarked as Taxotere® by Rhone-Poulenc Rorer now it is Sanofi Aventis) was approved by the FDA for the treatment of advanced ovarian cancer in April 1994 and in December 1999 for the treatment of patients with locally advanced or metastatic non-small cell lung cancer. Docetaxel is obtained by semisynthesis from 10-deacetylbaccatin III, non-cytotoxic precursor extracted from the needles of the European yew, Taxus baccata (Zamir et al., 1992). Taxotere is approximately twice as potent as taxol in inhibiting cold and calcium-induced depolymerization of microtubules (Gueritte et al., 1993).

In acidic media or in the presence of electrophilic agents, D ring as well as B ring opening and/or rearrangement will occur with docetaxel (Figure 10.1A) depending on the conditions employed. In basic media, the cleavage of ester groups at positions 2, 4, and/or 13 will occur. One of the principal paths of degradation is the epimerization of the hydroxyl group at position 7 which results in the formation of 7-epidocetaxel by way of retro aldol reaction. The degradation of docetaxel can results in products which have reduced activity or are completely inactive. They also demonstrate pharmacological and toxicological profiles completely different from the active drug (Machado et al., 2009).

Three main modifications of taxoids have been shown to occur when they are introduced in the organism: epimerization, hydrolysis, and hydroxylation. Among the three principal metabolic modifications of taxoids identified to date in the organism, hydroxylation appears to be the most important. The reversible epimerization of hydroxyl group at C-7 occurs with both paclitaxel and docetaxel. It seems to be more readily achieved in docetaxel, where it introduces additional complexity of the metabolites. The process, however, may be of minor pharmacological importance since it does not modify the cytotoxicity of the drug (Wright et al., 1995).

Using the HPLC/APCI-MS method, Monsarrat et al., characterized 7-epipaclitaxel in both plasma and urine of patients receiving a 3h infusion of paclitaxel (Monsarrat et al., 1994). In plasma, the maximal concentrations were observed at the end of the infusion period, reaching 2.3 to 6.0μM for paclitaxel and 0.11μM for 7-epipaclitaxel. Thus, the plasma concentration of 7-epipaclitaxel remained 20-55 fold lower than the concentration of paclitaxel.

Less than 2% of the total drug detected in the feces of patients treated with [^{14}C] docetaxel was in the form of 7-epidocetaxel (Vuilhorgne et al., 1994). A small amount of 7-epidocetaxel was also found in plasma extracts. The proportion of 7-epidocetaxel reached 2.6% in rat bile (Gillard et al., 1994). The 7-epidocetaxel was less cytotoxic than docetaxel in P-388 leukemia (IC_{50} 60 and 7ng/mL, respectively).

Czejka *et al.* recently reported, the quantification of 7-epidocetaxel in blood and urine of chemotherapy patients (Taxotere). In 8 of 12 patients 7-epidocetaxel was quantified in plasma at the end of infusion (concentrations ranging from 0.05μg/ml to 0.54μg/ml). In urine, 7-epidocetaxel has been found in 7 of 12 patients (ranged from 3.21μg/ml to 66.37μg/ml). These results indicate that there is significant amount 7-epidocetaxel

formation *in vivo* after Taxotere infusion (about 14% of injected dose) (Czejka et al., 2010).

The hydrolysis products of taxol have been isolated by high-performance liquid chromatography and identified by nuclear magnetic resonance and mass spectroscopy. In contrast to taxol, the major hydrolysis product, baccatin III, has little cytotoxic activity and does not promote *in vitro* microtubule assembly. In cell culture medium, the concentration of taxol decreases with time and 7-epitaxol, which exhibits properties comparable to those of taxol both on cells and on *in vitro* microtubuli polymerization, is formed. Baccatin III is found in small quantities in the cell medium, although it is barely detectable within the cells. It is concluded that 7-epitaxol is the major derivative of taxol found in cells and that its presence does not alter, in a major way, the overall biological activity of taxol (Ringel and Horwitz, 1987).

Paclitaxel and 7-epipaclitaxel show almost identical cytotoxic potencies. Ringel and Horwitz reported ED_{50} (drug concentration inhibiting cell division by 50% after 72hr) of paclitaxel and 7-epipaclitaxel to be 0.08μM and 0.12μM with mouse J744.2 cells, and 0.32μM and 0.31μM with CHO cells, respectively. However, against KB cells, the ED50 of 7-epipaclitaxel was three times higher than the ED_{50} of paclitaxel (Ringel and Horwitz, 1987).

Bornique and Lemarie (Bornique and Lemarie 2002) investigated the interactions of docetaxel and its epimer 7-epidocetaxel with recombinant human cytochrome P450 1B1 (hCYP1B1) which is present in various human tumors and is postulated to be responsible for the development of resistance of tumor cells toward chemotherapeutic agents, including docetaxel. The authors observed that at a concentration of 10μM, the 7-epidocetaxel increased the activity of hCYP1B1 by more than 7 fold, confirming that is a potent inducer of this enzyme. Hence, the presence of 7-epidocetaxel in the pharmaceutical formulations is responsible for the development of resistance of tumor cells to the active drug, docetaxel and/or its trihydrate. Therefore, it is desirable to minimize or eliminate the presence of 7-epidocetaxel in pharmaceutical formulations containing docetaxel and/or its trihydrate.

Taxotere one compartment composition containing a taxane derivative in association with ethanol and polysorbate 80 is proved to be fairly unstable (Sandoz, 2008). This composition shows a significant degradation, expressed in the formation of 7-epidocetaxel when exposing it to heating. The 7-epidocetaxel is a well known degradation product of the docetaxel (Vasu Dev et al., 2006). It would be therefore highly desirable to develop alternative compositions having an improved stability.

As per the invention of Sandoz (Sandoz, 2008), it has surprisingly found that, the addition of an organic and/or inorganic acid induces a significant increase in stability of the composition even after heating at higher temperature. According to the invention the acid is selected in the group consisting of citric acid, acetic acid, formic acid, ascorbic acid, aspartic acid, benzoic acid, hydrochloric acid, sulphuric acid, phosphoric acid, tartaric acid, glutamic acid, lactic acid, maleic acid or succinic acid. Most preferred acid is

citric acid. Especially preferred acid is citric acid anhydrous as defined in the European Pharmacopoeia 2007.

The Sun Pharmaceutical Industries obtained marketing authorisation for Docefrez® by European Medicines Agency. In the application they have showed the acute toxicity of Docefrez® containing 1% 7-epimer impurity was comparable to Taxotere.

The objective of the present research was to prepare formulations of docetaxel impurities, as like marketed Taxotere and to evaluate their *in vitro* cytotoxicity behaviour against human adenocarcinoma cell line (A549) and mouse melanoma cell line (B16F10), and *in vivo* acute toxicity in B16F10 pulmonary metastasis bearing mice at single dose of 40mg/kg Taxotere containing 10% of docetaxel impurities, 7-epidocetaxel and 10-oxo-7-epidocetaxel, separately. Also, the objective was to further evaluate their *in vitro* anti-metastatic character (using scratch wound assay), interaction with matrix metalloproteinases (MMP9 and MMP2), and effect on apoptosis and cell cycle.

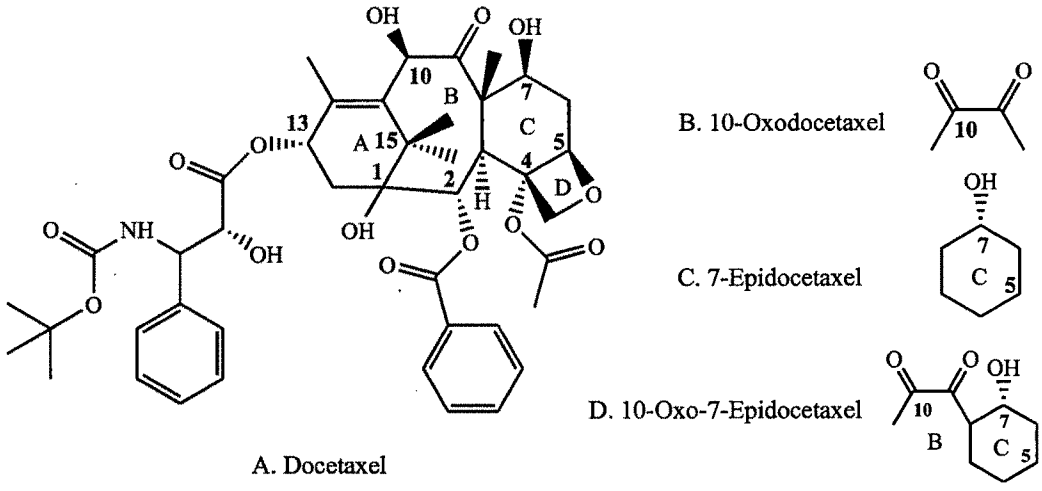


Figure 10.1. Chemical structures of A. Docetaxel and its degradation impurities B. 10-Oxodocetaxel, C. 7-Epidocetaxel and D. 10-Oxo-7-Epidocetaxel

10.2. Materials and Methods

The docetaxel and docetaxel impurities, 7-epidocetaxel and 10-oxo-7-epidocetaxel (96% purity), were obtained as gift samples from Fresenius Kabi Oncology Limited, GURGAON, India. Tween-80 was purchased from Sigma Aldrich, Mumbai, India.

Cell culture

B16F10, a mouse melanoma cell line and A549, human lung adenocarcinoma cell line were purchased from NCCS, Pune, India. The B16F10 and A549 cell lines were maintained in Iscove's Modified Dulbecco's Media (IMDM) and RPMI-1640 (GIBCO),

respectively. The media was supplemented with 10% heat inactivated foetal bovine serum, FBS (GIBCO), and antibiotics (100U/mL penicillin and 100μg/mL streptomycin). Cultures were maintained at 37 ^{0}C in 5% CO_2 humidified atmosphere.

10.3. Preparation of docetaxel impurities formulation

The docetaxel impurities were formulated as like marketed Taxotere (Docetaxel for injection). The vehicle ethanol and tween-80 mixture (50:50, v/v) containing citric acid (2mg/mL) was prepared. The docetaxel impurities, 7-epidocetaxel, and 10-oxo-7-epidocetaxel were dissolved in the prepared vehicle at a concentration of 20mg/mL. The further dilutions were made with phosphate buffer saline (pH 4, adjusted with citric acid) to 0.5mg/mL solution and stored at 2-8 ^{0}C for 3 months. Also, the formulations were diluted to 0.5mg/mL with culture medium (IMDM) containing 10% foetal bovine serum and no citric acid, and stored at 2-8 ^{0}C for 3 months.

10.4. *In vitro* cytotoxicity assay

The cytotoxicity of Taxotere and docetaxel impurities 7-epidcoetaxel and 10-oxo-7-epidocetaxel, against A549 (human lung adenocarcinoma) and B16F10 (mouse melanoma) cell lines was determined using MTT dye reduction assay (Twentyman and Luscombe, 1987). Briefly, 4×10^3, 2×10^3, and 1.5×10^3 cells in exponential phase were seeded into 96-well plates for 24hr, 48hr, and 72hr respectively. The cells were incubated at 37 ^{0}C in a 5% CO_2 incubator for 24 h, during which the cells were attached and resumed to grow. The formulations were diluted with culture media to make serial concentrations of 0.01nM, 0.1nM, 1nM, 10nM, 100nM, 1μM, 10μM, and were added to wells in quadruplicate (100μL each). Control wells were treated with equivalent volumes of docetaxel free media. After 24h, 48h, and 72h, the supernatant was removed and each well was washed twice with 100μL of PBS. The 20μL of MTT (5mg/mL solution in PBS pH 7.4) and 80μL of culture medium were added to each well and incubated for overnight. The plates were then centrifuged for 20 minutes at 1500rpm (Rota 4R-V/F_M; Plasters Crafts Industries Ltd., Mumbai, India) and the unreduced MTT and medium were then discarded. The 100μL of DMSO was then added to dissolve the MTT formazan crystals. Plates were shaken for 2min and absorbance was read at dual wavelengths of 540/690nm as excitation and reference using the microplate reader (Molecular Devises, SPECTRUM AX190). The IC_{50} values were then calculated graphically (logarithmic scale) by plotting concentrations versus % viability. The % viability was calculated by considering the optical density of the control well as 100% viable (Sharma et al., 1996).

10.5. Anti-metastatic activity by wound scratch assay

The wound healing assay was performed as per the earlier reports (Goel and Gude 2011; Pichot et al., 2009; Lee et al., 2008). Cells grown in 12 well plates were treated with 1μg/ml mitomycin-C for 2hr. The cells were then lined off using a sterile tip, and the wells were washed to ensure that the wound area is devoid of cells. The cells were incubated with 2mL of 2nM (sub-toxic dose) solution of Taxotere, 7-epidocetaxel, and

10-oxo-7-epidocetaxel at 37 ^{0}C in a 5% CO_2 incubator for 24h. The drug solutions were removed, cells were washed twice with PBS, fixed using 70% methanol and photographed using Axiovert200 inverted microscope (Zeiss). The reference well cells were fixed immediately after wound was made and the control well cells were incubated with 2mL drug free media. The wound width was measured using the Carl Zeiss Axiovision Rel 4.8.2 imaging software and the results were presented as percent wound covered by considering the control group width as 100% wound covered.

10.6. Gelatin zymography

Sub-confluent plates of A549 and B16F10 cells were treated with 3mL of 2nM (non-toxic doses) solution of TXT, 7-epidocetaxel and 10-oxo-7-epidocetaxel for 24hr. The drug solutions were removed and cells were washed twice with PBS to remove serum traces. The 3mL of serum free DMEM was then added to each plate and the condition media was collected after 24hr. The media was concentrated using 30kDa cut-off filters from Millipore and normalised as per the individual cell counts. In addition, the condition medium collected from HT-1080 was also processed and used as a positive control representing MMP-9 (92kDa) and MMP-2 (72kDa). To assess the gelatinase activity, the samples (40μL) were incubated in sample buffer for 10 minutes at room temperature and run on 10 % SDS-PAGE containing 0.1% gelatin (w/v) as a substrate. After electrophoresis, the gel was washed twice in rinsing buffer (2.5% Triton X-100) for 15 minutes each and then incubated in developing buffer (Tris 50mM, $CaCl_2$ 100mM, $ZnCl_2$ 10μM, Triton 2.5% and NaN_3 0.02%) for at least 24 h. The gel was stained with 0.25% commassie brilliant blue R-250 and then destained (Water: Methanol: Acetic acid; 5:4:1, v/v) till the bands appeared. The gelatinase activity was visible as clear white zones in a dark background. Gel image was taken in the gel documentation machine and densicot analysis of the band intensity was done (Wang et al., 2009).

10.7. Apoptosis assay using FACS

Sub-confluent plates were treated with 1mL of 2nM, 10nM and 25nM solutions of Taxotere, 7-epidocetaxel, and 10-oxo-7-epidocetaxel for a period of 24hr and 48hr. Cells were harvested, mixed with supernatant containing apoptic cells, washed twice with PBS and fixed with 70% chilled ethanol. The cells were washed twice with PBS and then resuspended in 200μl PBS containing 10μl RNAse (0.5 mg/ml) and incubated for 30minutes at 37 ^{0}C. The volume was then made up to 1mL and 50μl propidium iodide (PI) (50μg/ml) was added. Acquisition was done on FACS Calibur. At least 10000 events were collected and then analysed using CellQuest software (Salma and McDermott, 2012; Kanzawa et al., 2003).

10.8. Cell cycle analysis

The protocol is same as discussed in apoptosis study. The 10000 events were collected and analysed for cell cycle using ModFit LT V2.0 (PMac) software.

10.9. Acute toxicity study

Animals

Female C57BL/6 mice (6-8 weeks old), ranging from 18 to 22g were provided by Animal Care Facilities, ACTREC, TATA Hospital, Mumbai. All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee. All care and handling of animals were performed with the approval of institutional review board of animal experiments.

Method

An *in vivo* study in B16F10 melanoma lung metastasis mice model was performed to compare the acute toxicity, after a single i.v. dose, of Taxotere injection alone and with 10% 7-epidocetaxel and 10-oxo-7-epidocetaxel impurities. The 6 to 8 week-old female C57BL/6 mice were injected with 0.1 million cells/100ul PBS intravenously. Mice were randomly assigned into 4 groups (6mice/group): (A) Untreated control (PBS); (B) Taxotere treated; (C) Taxotere with 10% 7-epidocetaxel treated; and (D) Taxotere with 10% 10-oxo-7-epidocetaxel treated. The mice were dosed at 40mg/kg (approximately equivalent to human maximum tolerance dose) of Taxotere and Taxotere containing 10% impurities separately on 9th day after B16F10 cell inoculation. The 7-epidocetaxel and 10-oxo-7-epidocetaxel (formulated as like Taxotere injection) equivalent to 10% were mixed separately with Taxotere and injected to mice intravenously. The animals were weighted at every second day throughout the experiment and the dead ones were recorded. These data were used as indicators of toxicity of Taxotere alone and with docetaxel impurities. The mice were sacrificed on 20th day and the lungs were excised, photographed, and fixed in 10% formaldehyde solution until use. The lung sections were fixed, stained with hematoxylin-eosin and observed under phase contrast microscope (Axio Imager. Z1 upright microscope, Germany).

10.10. Therapeutic study of 10-oxo-7-epidocetaxel

An *in vivo* study in B16F10 melanoma lung metastasis bearing mice was performed to determine the therapeutic effectiveness of docetaxel impurity, 10-oxo-7-epidocetaxel, in lung tumor growth/metastasis inhibition. The 6 to 8 week old female C57BL/6 mice were injected with 0.1 million cells/100ul PBS intravenously. Mice were randomly assigned into 2 groups (6mice/group): (A) Untreated control (PBS); (B) 10-oxo-7-epidocetaxel treated. On 9th day of B16F10 cell inoculation the mice were injected intravenously with 10-oxo-7-epidocetaxel at single dose of 20mg/kg (400μg/100μL/Mouse). The 10-oxo-7-epidocetaxel was formulated as like Taxotere injection (dissolved in ethanol: polysorbate-80; 50:50, v/v mixture at the concentration of 20mg/mL). The further dilutions were made in sterile PBS and injected to mice *via* tail vein. The 100μL PBS was injected through tail vein to control group mice. The animals were weighted at every second day throughout the experiment and the dead ones were recorded. These data were used as indicators of toxicity of 10-oxo-7-epidocetaxel. The mice were sacrificed on 20th day and the lungs were excised, photographed, the numbers of surface metastatic nodules were counted, and fixed in

10% formaldehyde solution until use. The lung sections were fixed, stained with hematoxylin-eosin and observed under phase contrast microscope (Axio Imager. Z1 upright microscope, Germany).

10.11. Results and Discussion

10.11.1. Stability of docetaxel impurities

The actual concentration of impurities, 7-epidocetaxel and 10-oxo-7-epidocetaxel, present in the prepared formulations was determined by RP-HPLC method, as described before, using standard calibration curves of individual impurities. The characteristic RP-HPLC peaks of docetaxel and impurities are shown in Figure 10.2.

Ringel and Horwitz (Ringel and Horwitz, 1987) determined the epimerization of paclitaxel in the medium in the presence or absence of cells. Moreover, this process was reversible, since 7-epipaclitaxel was partially epimerized to paclitaxel when incubated in the medium. In the present study, we stored the docetaxel impurities in IMDM culture medium containing 10% foetal bovine serum, to study the stability of these impurities and the reversible epimerisation of these impurities to docetaxel and also the formation of other impurities. From these results we can predict stability of these impurities in circulation.

After 3 months of storage at 2-8 ^{0}C in IMDM culture medium containing 10% foetal bovine serum, impurities remained soluble and no precipitates were observed. It is very clear from the HPLC chromatogram (Figure 10.3A) that, about more than 50% 7-epidocetaxel (RT: 6.22 minute) was converted into docetaxel (RT: 4.71 minute) during the storage of 3 months. This confirms the reversible epimerisation of 7-epidocetaxel into docetaxel in the medium. In the same chromatogram we can also observe the formation of 10-oxo-7-epidocetaxel (RT: 6.94 minute) and other impurities. The chromatogram (Figure 10.3B) showed more intense and distinct peak for 10-oxo-7-epidocetaxel (RT: 6.95 minute) than docetaxel (RT: 4.71 minute), and other impurities (at RT: 5.03 and 5.4 minute). The peak corresponding to 7-epidocetaxel (at RT: 6.22 minute) was not found and this indicates no/less conversion of 10-oxo-7-epidocetaxel to 7-epidocetaxel. The peak at RT of 5.48 minute might correspond to 10-oxodocetaxel. These results clearly indicates more stability of 10-oxo-7-epidocetaxel in the medium than 7-epidocetaxel after 3 months of storage. Therefore the 10-oxo-7-epidocetaxel would remain more stable in the circulation than 7-epidocetaxel and docetaxel.

The gifted impurities were reported to have ~96% purity. To determine the stability of impurity formulations during storage, we stored these impurities in phosphate buffer saline of pH 4 adjusted with citric acid at 2-8 ^{0}C for 3 months. After 3 months of storage the stability of impurities was analysed using HPLC method. The Figure 10.4A and 10.4B shows peaks corresponding to 7-epidocetaxel (RT: 6.69 minute) and 10-oxo-7-epidocetaxel (RT: 7.843 minute), respectively. The chromatograms shows no significant formation of docetaxel and other impurities as does in IMDM culture medium indicating more stability of these impurities at pH 4 during storage. The impurities remained

soluble and no precipitates were observed after 3 months of storage. The peak at RT of 6.78 minute in the Figure 10.4B was correspond to 7-epidocetaxel and this might be due to epimerisation and/or due to the presence of 7-epimer in the standard sample (96% purity).

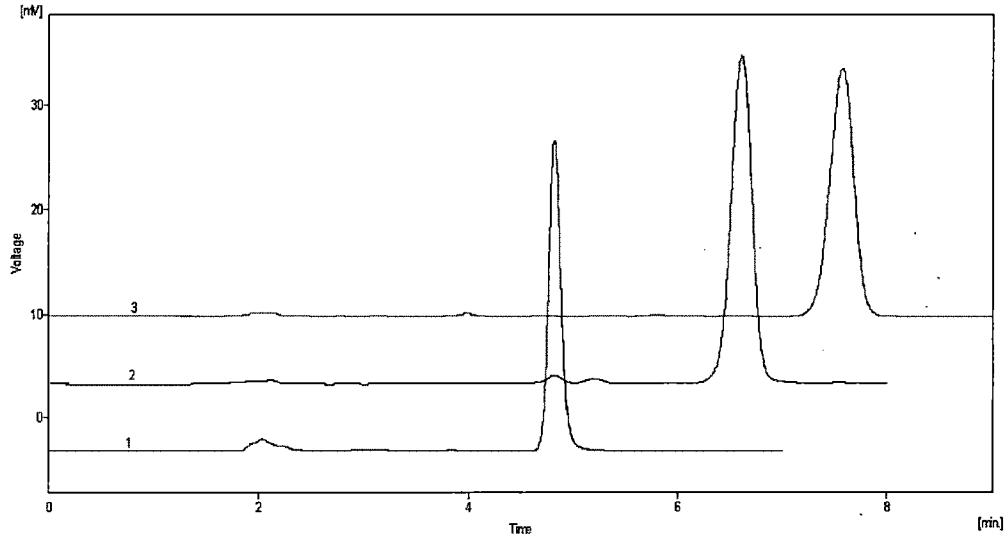


Figure 10.2. Characteristic peaks of (1). DTX (RT: 4.82±0.076 minute; RRT: 1) (2). 7-epidocetaxel (RT: 6.58±0.054 minute; RRT: 1.366) and (3). 10-oxo-7-epidocetaxel (RT: 7.57±0.042 minute; RRT: 1.571).

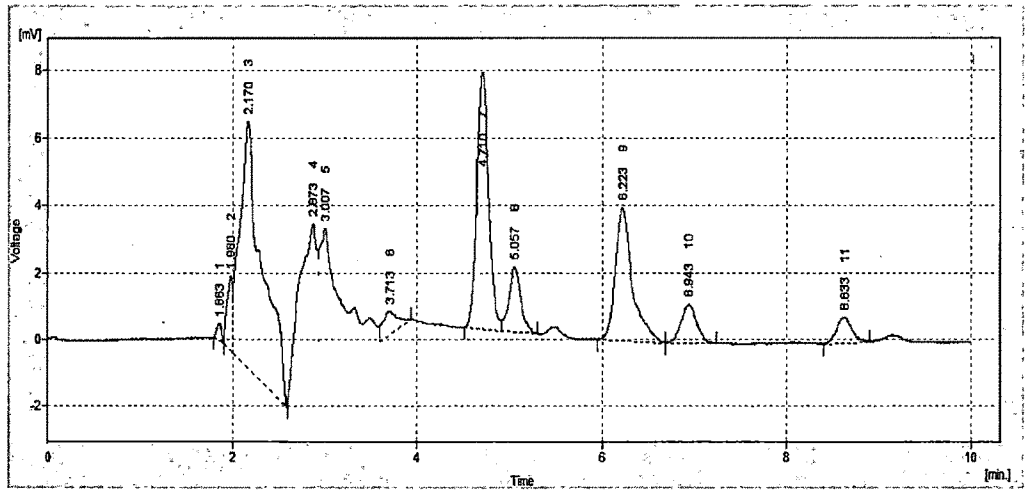


Figure 10.3A. The HPLC chromatogram of 7-epidocetaxel after 3 months of storage at 2-8 ^{0}C in IMDM medium containing no citric acid

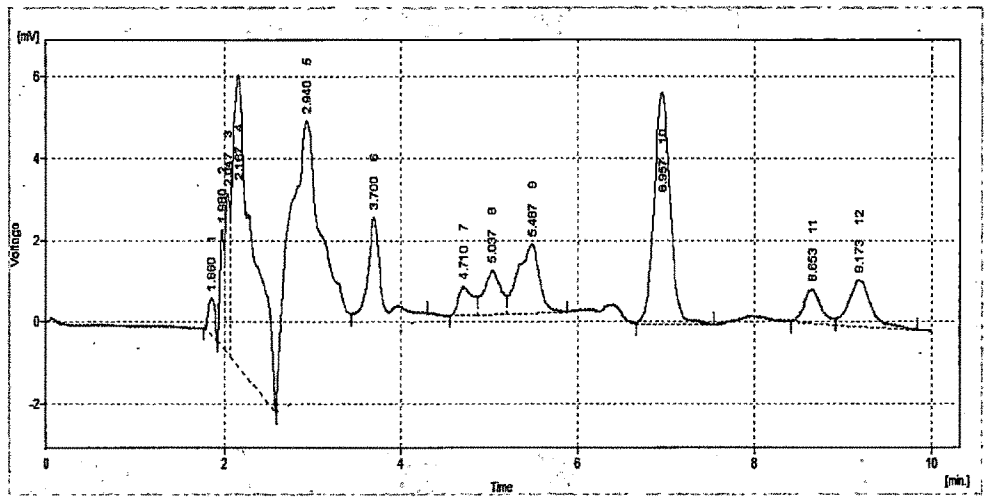


Figure 10.3B. The HPLC chromatogram of 10-oxo-7-epidocetaxel after 3 months of storage at 2-8 °C in IMDM medium containing no citric acid

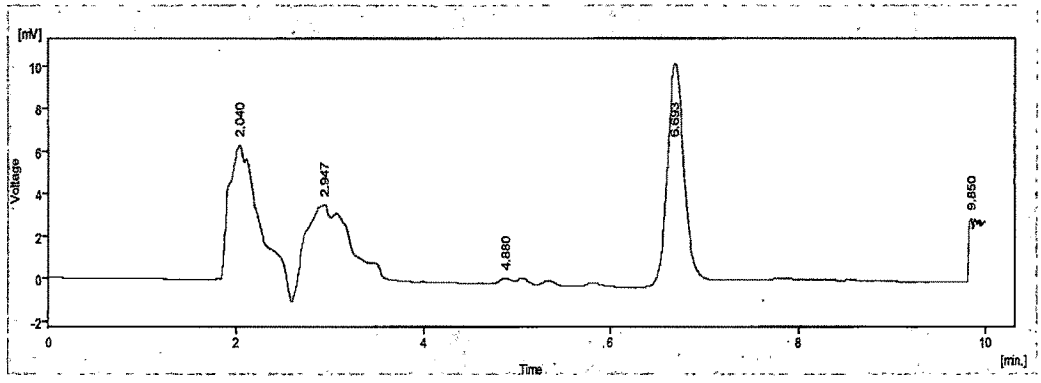


Figure 10.4A. The HPLC chromatogram of 7-epidocetaxel after 3 months of storage at 2-8 °C in PBS (pH 4, adjusted with citric acid)

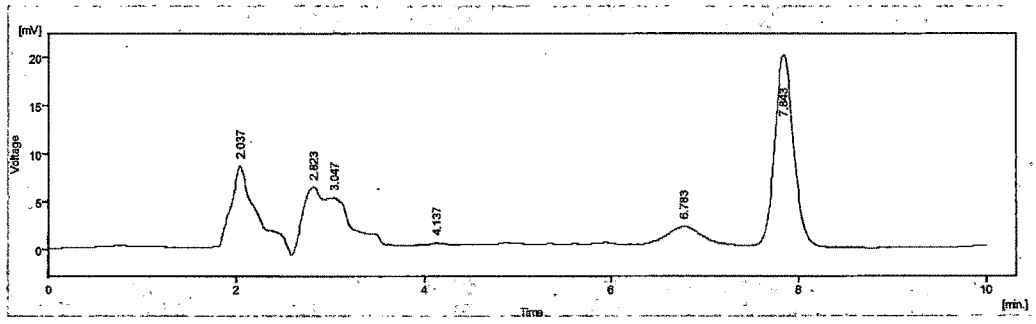


Figure 10.4B. The HPLC chromatogram of 10-oxo-7-epidocetaxel after 3 months of storage at 2-8 °C in PBS (pH 4, adjusted with citric acid)

10.11.2. *In vitro* cytotoxicity study

We evaluated the cytotoxic effects of commercial docetaxel formulation Taxotere and DTX impurities, 7-epidocetaxel and 10-oxo-7-epidocetaxel, against human lung adenocarcinoma cell line (A549) and mouse melanoma cell line (B16F10). Cells were exposed to the drug formulations for 24h, 48h, and 72h and cell viability was determined. All formulations resulted in concentration dependent and time dependent inhibition of the proliferation of A549 (Figure 10.5) and B16F10 cells (Figure 10.6).

A quantitative evaluation of the *in vitro* therapeutic effect of a dosage form is IC_{50}, which is defined as the drug concentration needed to kill 50% of the incubated cells in a designated time period. The IC_{50} value after 24h treatment of A549 cells is found high for 10-oxo-7-epidocetaxel (140±20nM) than Taxotere (25±4.08nM) and 7-epimer (6.5±1.91nM). The Taxotere is about 5.6 times more cytotoxic than 10-oxo-7-epidocetaxel and 3.84 times less cytotoxic than 7-epidocetaxel after 24hr treatment. After 48hr and 72hr of treatment the IC_{50} values were decreased for all formulations (Table 10.1) as compared to 24h. The Taxotere showed high cytotoxicity than 7-epidocetaxel and 10-oxo-7-epidocetaxel. The 10-oxo-7-epidocetaxel remained less cytotoxic than Taxotere and 7-epidocetaxel after 24hr, 48hr, and 72hr treatment. However, it showed 657 times and 129 times increased cytotoxicity after 48hr and 72hr treatment, respectively, as compared to 24hr treatment. Therefore the 10-oxo-7-epidocetaxel showed less cytotoxicity after 24hr and showed high cytotoxicity with increased time (after 48 and 72hr treatment). The 10-oxo-7-epidocetaxel showed about 8.76 times and 9.62 times less cytotoxicity after 48 and 72hr treatment, respectively as compared to 7-epidocetaxel. Similarly, the 10-oxo-7-epidocetaxel showed about 41.76 and 216.6 times less cytotoxicity after 48 and 72hr treatment, respectively as compared to Taxotere. The 7-epidocetaxel showed about 4.76 and 22.5 times less cytotoxicity after 48hr and 72hr treatment, respectively as compared to marketed Taxotere. Therefore from our results we can conclude that, the 7-epimer showed more cytotoxicity against A549 cells than Taxotere after 24hr treatment but remain less cytotoxic than Taxotere after 48 and 72hr treatment. The 10-oxo-7-epidocetaxel showed less cytotoxic than both Taxotere and 7-epimer after 24hr, 48hr, and 72hr of treatment. Therefore the 10-oxo-7-epidocetaxel was found less cytotoxic against A549 cell line as compared to 7-epidocetaxel and marketed Taxotere.

In case of B16F10 cells the IC_{50} values were shifted to higher values after 24hr, 48hr and 72hr of treatment as compared to A549 cells indicating B16F10 melanoma cells are less sensitive than A549 cells. The Taxotere showed more cytotoxicity than 7-epidocetaxel and 10-oxo-7-epidocetaxel. After 48hr treatment the Taxotere showed about 1.2 times and 12.58 times more cytotoxicity as compared to 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively. After 72hr treatment the Taxotere showed about 5.88 times and 85.49 times more cytotoxicity as compared to 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively. Against both the cell lines the marketed Taxotere showed low IC_{50} values (more cytotoxicity) than 7-epidocetaxel and 10-oxo-7-epidocetaxel.

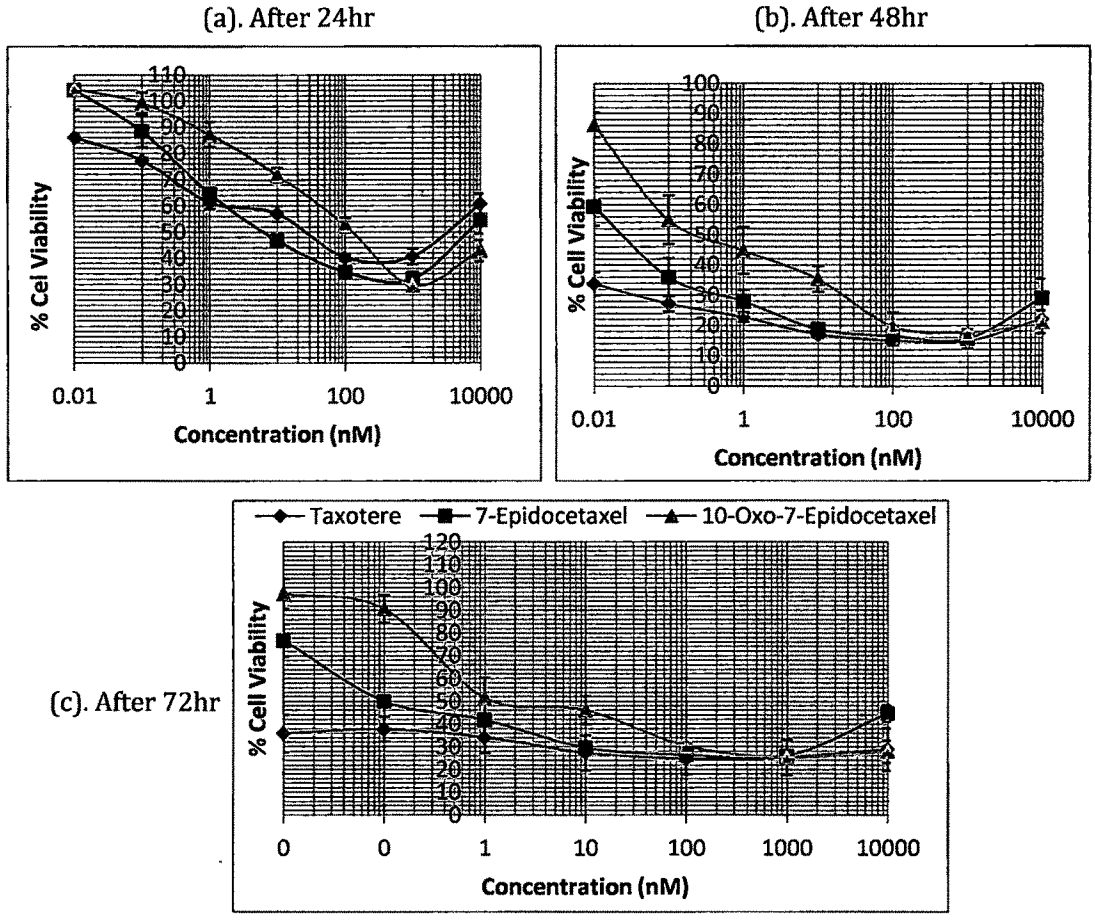
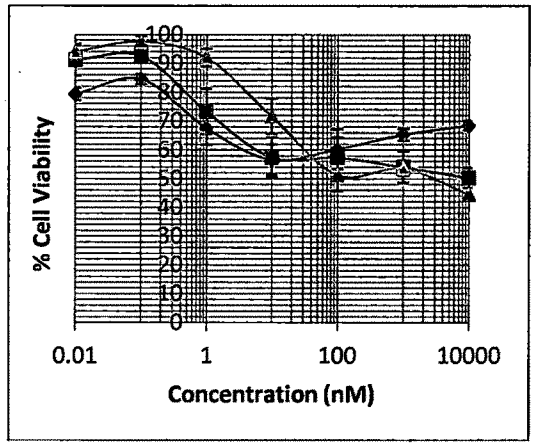
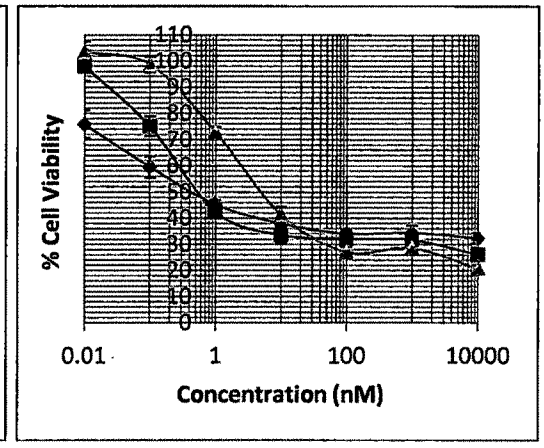


Figure 10.5. The % A549 cell viability after (a). 24hr, (b). 48hr and (c). 72hr of treatment with Taxotere, 7-Epidocetaxel, and 10-oxo-7-epidocetaxel (♦: Taxotere; ■: 7-epidocetaxel; ▲: 10-oxo-7-epidocetaxel).

(a). After 24hr



(b). After 48hr



(c). After 72hr

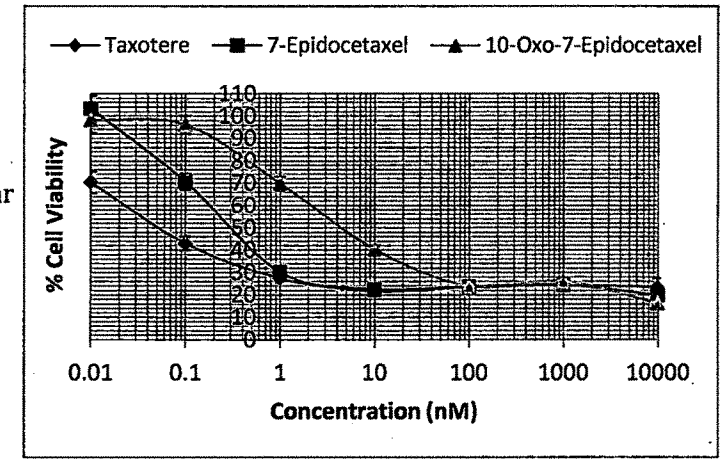


Figure 10.6. The % B16F10 cell viability after (a). 24hr, (b). 48hr and (c). 72hr of treatment with Taxotere, 7-Epidocetaxel, and 10-oxo-7-epidocetaxel (♦: Taxotere; ■: 7-epidocetaxel; ▲: 10-oxo-7-epidocetaxel).

Table 10.1. The comparison of the IC_{50} values (nM) of Taxotere, 7-epidocetaxel and 10-Oxo-7-epidocetaxel against A549 and B16F10 cells at different time points

Drug	A549		
	24 hr	**48hr**	**72hr**
Taxotere	25±4.08	0.0051±0.0017	0.005±0.001
7-Epidocetaxel	6.5±1.91	0.0243±0.01	0.1125±0.059
10-Oxo-7-Epidocetaxel	140±20	0.213±0.056	1.083±0.52
	B16F10		
Taxotere	not found	0.425±0.17	0.0512±0.01
7-Epidocetaxel	2500±866	0.512±0.08	0.3±0.05
10-Oxo-7-Epidocetaxel	3000±1471	5.35±0.46	4.36±0.16

Values are Mean±SD, n=3.

10.11.3. Anti-metastatic activity by wound scratch assay

Migration is one of the key features in cancer progression involved in dissemination of tumor cells from its origin to a distant site (Friedl and Wolf, 2003). Rho GTPases are one of the key determinants involved in regulating migration by affecting actin cytoskeleton as well as influencing microtubular network (Etienne-Manneville and Hall, 2002). Microtubules consequently tone the activities of these Rho GTPases as well (Watanabe et al., 2005). Docetaxel has been shown to inhibit Cdc-42, one of the key members of Rho GTPase family in head and neck cancers (Kogashiwa et al., 2010). Further, docetaxel also inhibits endothelial cell migration (Murtagh et al., 2006; Grant et al., 2003). Based on these reports we have tested the *in vitro* anti-migratory effect of Taxotere and docetaxel impurities 7-epidocetaxel, and 10-oxo-7-epidocetaxel by wound scratch assay, against human lung adenocarcinoma cell line A549 (Figure 10.7) and mouse melanoma cell line B16F10 (Figure 10.8). According to this assay the anti-migratory effect of a drug is indirectly proportional to % wound covered as compared to untreated cells. The lesser the wound covered in treated cells as compared to untreated cells indicates that the drug has a good anti-migratory effect against the tested cell lines.

Against A549 cells we observed about 60.5±3.01%, 41.92±3.11%, and 23.94±5.44% of covered wounds at 2nM (sub-toxic dose) of Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively (Figure 10.9A). About 39.5%, 58% and 76% *in vitro* anti-metastatic activity was found with Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively. Surprisingly, we have observed about 1.46 fold (17.5% increased activity) and 1.92 fold (36.5% increased activity) increase in *in-vitro* anti-metastatic activity with 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively as compared to marketed Taxotere.

Further, to confirm the results obtained against A549 cells, we performed the same assay against highly metastatic mouse melanoma cell line B16F10. We observed about 64.24±8.46%, 79.01±11.1%, and 47.7±12.04% of covered wounds at 2nM (sub-toxic dose) of Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively (Figure 10.9B). About 36%, 21% and 52% *in vitro* anti-metastatic activity was found with Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively. The 7-epidocetaxel showed 17% decreased anti-metastatic activity than marketed Taxotere against B16F10 cells but the 10-oxo-7-epidocetaxel showed about 16% increased anti-metastatic activity than marketed Taxotere. The 7-epidocetaxel showed mixed effect against A549 (increased anti-metastatic activity than Taxotere) and B16F10 (decreased anti-metastatic activity than Taxotere). Whereas, the 10-oxo-7-epidocetaxel showed increased anti-metastatic activity against both the cell lines tested as compared to Taxotere. Our study truly identified a novel role of impurities specially 10-oxo-7-epidocetaxel as a potent inhibitor of cell migration. This is a preliminary observation being noticed as per now but the further investigations are needed to address the potential of 10-oxo-7-epidocetaxel in this era of cancer therapeutics.

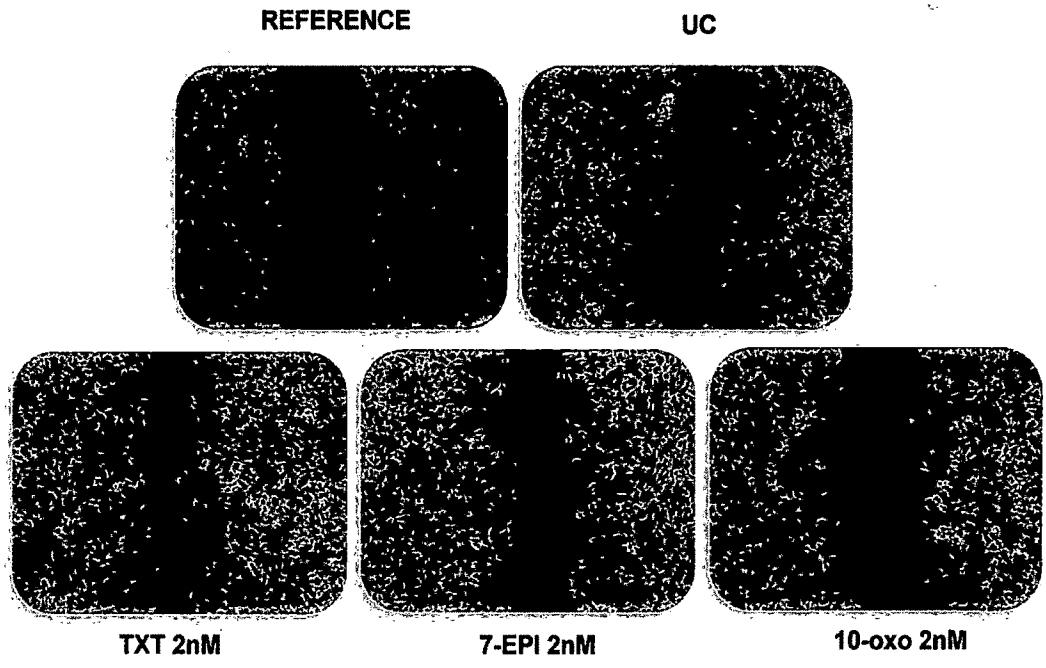


Figure 10.7. Zeiss Axio Inverted Microscopic images showing migration of A549 cells in the presence of 2mL of 2nM concentration of Taxotere (TXT), 7-epidocetaxel (7-EPI), and 10-oxo-7-epidocetaxel (10-oxo).

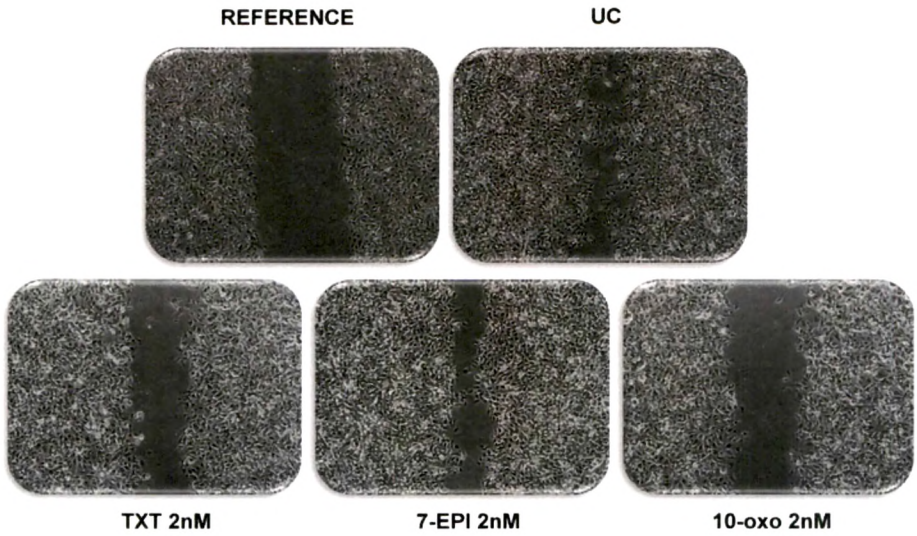


Figure 10.8. Zeiss Axio Inverted Microscopic images showing migration of B16F10 cells in the presence of 2mL of 2nM concentration of Taxotere (TXT), 7-epidocetaxel (7-EPI), and 10-oxo-7-epidocetaxel (10-oxo).

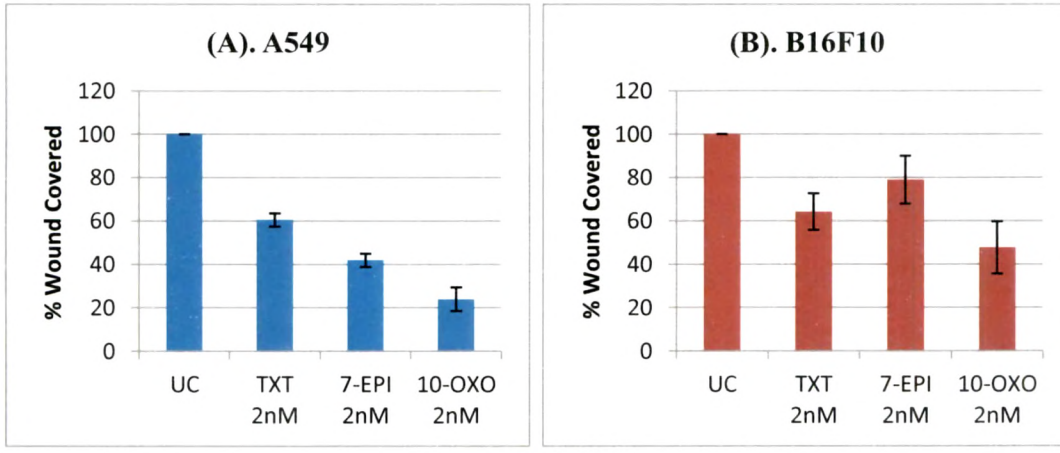


Figure 10.9. The % wound covered in the presence of 2mL of 2nM concentration of Taxotere (TXT), 7-epidocetaxel (7-EPI), and 10-oxo-7-epidocetaxel (10-oxo). Values represented are Mean±SD, n=3. The Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel showed significant anti-metastatic activity, against A549 cells, as compared to untreated control cells ($p<0.0001$). The 10-oxo-7-epidocetaxel showed significantly increased anti-metastatic activity, against A549 cells, as compared to Taxotere ($p<0.0001$) and 7-epidocetaxel ($p<0.001$). The 7-epidocetaxel showed significantly increased anti-metastatic activity as compared to Taxotere ($p<0.001$).

Similarly, the Taxotere ($p<0.01$) and 10-oxo-7-epidocetaxel ($p<0.001$) showed significant anti-metastatic activity, against B16F10 cells, as compared to untreated control cells. The 10-oxo-7-epidocetaxel showed significantly increased anti-metastatic activity, against B16F10 cells, as compared to 7-epidocetaxel ($p<0.05$, $p=0.019$).

10.11.4. Gelatin zymography

Matrix metalloproteinases (MMPs) are one of the major key players in the process of tumor development (Polette et al., 2004; Kessenbrock et al., 2010). Among the major families of MMPs, MMP-2 and MMP-9 are the major gelatinases (Egeblad and Werb, 2002). Gelatin is a by product of collagen which is one of the major constituents of basement membrane (Pöschl et al., 2004). Thus, for tumor cells to escape into the bloodstream they need to degrade this barrier of basement membrane.

Therefore, in the present study, we have tested the interaction of docetaxel, 7-epidocetaxel, and 10-oxo-7-epidocetaxel with MMPs of human adenocarcinoma cell line (A549) and mouse melanoma cell line (B16F10) to determine their role in tumor invasion and metastasis.

The human A549 cells showed higher activity of MMP-2 than MMP-9 (Figure 10.10A). This result was in accordance with previous findings which clearly revealed that the MMP-2 is a more predictable marker of lung cancer confirmed by immunostaining and elevated serum levels (Guo et al., 2007). The mouse B16F10 melanoma cells showed higher levels of MMP-9 and very weak bands were observed for MMP-2 (Figure 10.10B). It has been reported that the higher serum levels of MMP-9 in melanoma is inversely proportional to patients survival rate (Nikkola et al., 2005).

The zymogram of A549 and B16F10 cells treated with docetaxel, 7-epidocetaxel, and 10-oxo-docetaxel showed no changes in the activity of both endopeptidases (Figure 10.10A and 10.10B). The densitometry analysis of bands also showed negligible changes in band intensities of docetaxel and docetaxel impurities treated cells as compared to untreated sample. These results clearly suggest that docetaxel and its impurities do not affect the activity of gelatinases. However, they might affect other molecules involved in the processes invasion, but it needs to be further elucidated.

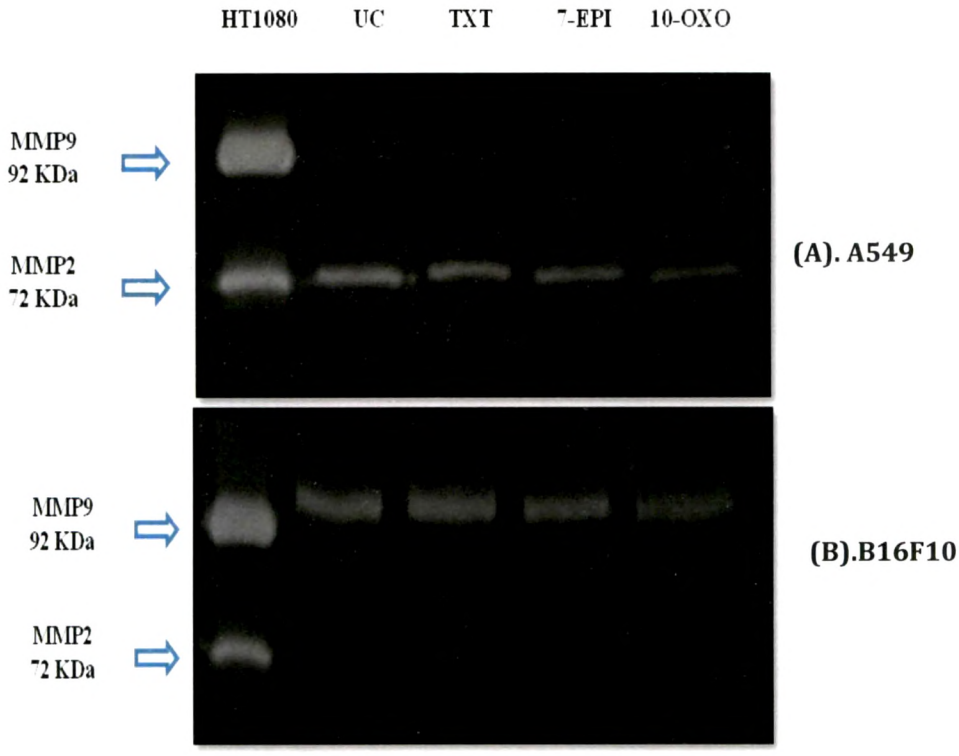


Figure 10.10. The comparison of interaction of Taxotere (TXT), 7-epidocetaxel (7-EPI) and 10-oxo-7-epidocetaxel (10-oxo) with MMP-9 and MMP-2 of (A). A549 cells and (B) B16F10 cells to determine their role in tumor invasion and metastasis.

10.11.5. Apoptosis study by FACS

By staining the cells with propidium iodide (PI), FACS was used to distinguish and quantitatively determine the percentage apoptotic cells. At least 10000 events were collected and analysed using CellQuest software. The % A549 and B16F10 apoptotic cells gated (M_1), after treatment with 2nM, 10nM, and 25nM of Taxotere, 7-epidocetaxel, and 10-oxo-7-epidocetaxel for a period of 24hr and 48hr, was shown in Figure 10.11 and Figure 10.12, respectively.

The increased % apoptosis of A549 cells and B16F10 cells was observed with increase in concentration of Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel from 2nM to 25nM (Table 10.2). About 4.86 and 10.08 fold increased apoptosis of A549 cells was observed with Taxotere as compared to 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively at 2nM concentration after 24hr of treatment. This indicates Taxotere is highly cytotoxic at low concentration as compared to docetaxel impurities tested. At 10nM concentration we observed no significant difference in apoptosis caused by Taxotere (42.57±1.89%), 7-epidocetaxel (39.18±1.52%) and 10-oxo-7-epidocetaxel (34.82±0.78%). At 10nM and 25nM both Taxotere and 7-epidocetaxel caused almost equal apoptosis as compared to 10-oxo-7-epidocetaxel (Table 10.2) which caused less apoptosis than Taxotere and 7-epidocetaxel at all concentrations tested. The similar kind of result was obtained after 48hr treatment (Table 10.2 and Figure 10.13A).

Against B16F10 cells, we observed the apoptosis was concentration and time dependent. The apoptosis increased with increase in the concentrations (from 2nM to 25nM) and treatment time (from 24hr to 48hr) (Table 10.2 and Figure 10.13B). The Taxotere and impurities showed almost similar % apoptosis at 2nM concentration after 24hr and 48hr treatment. The 10-oxo-7-epidocetaxel caused % apoptosis similar to 7-epimer but caused 5.97% and 7.77% less apoptosis at 10nM and 25nM, respectively than Taxotere after 24hr of treatment. The 10-oxo-7-epidocetaxel caused apoptosis similar to Taxotere and about 5.52% increased apoptosis than 7-epidocetaxel at 25nM and after 48hr of treatment.

We observed decreased B16F10 cell apoptosis as compare to A549 cells at almost all concentrations tested and after 24hr and 48hr treatments. This clearly indicates that the B16F10 cells are less sensitive to Taxotere and impurities as compared to A549 cells.

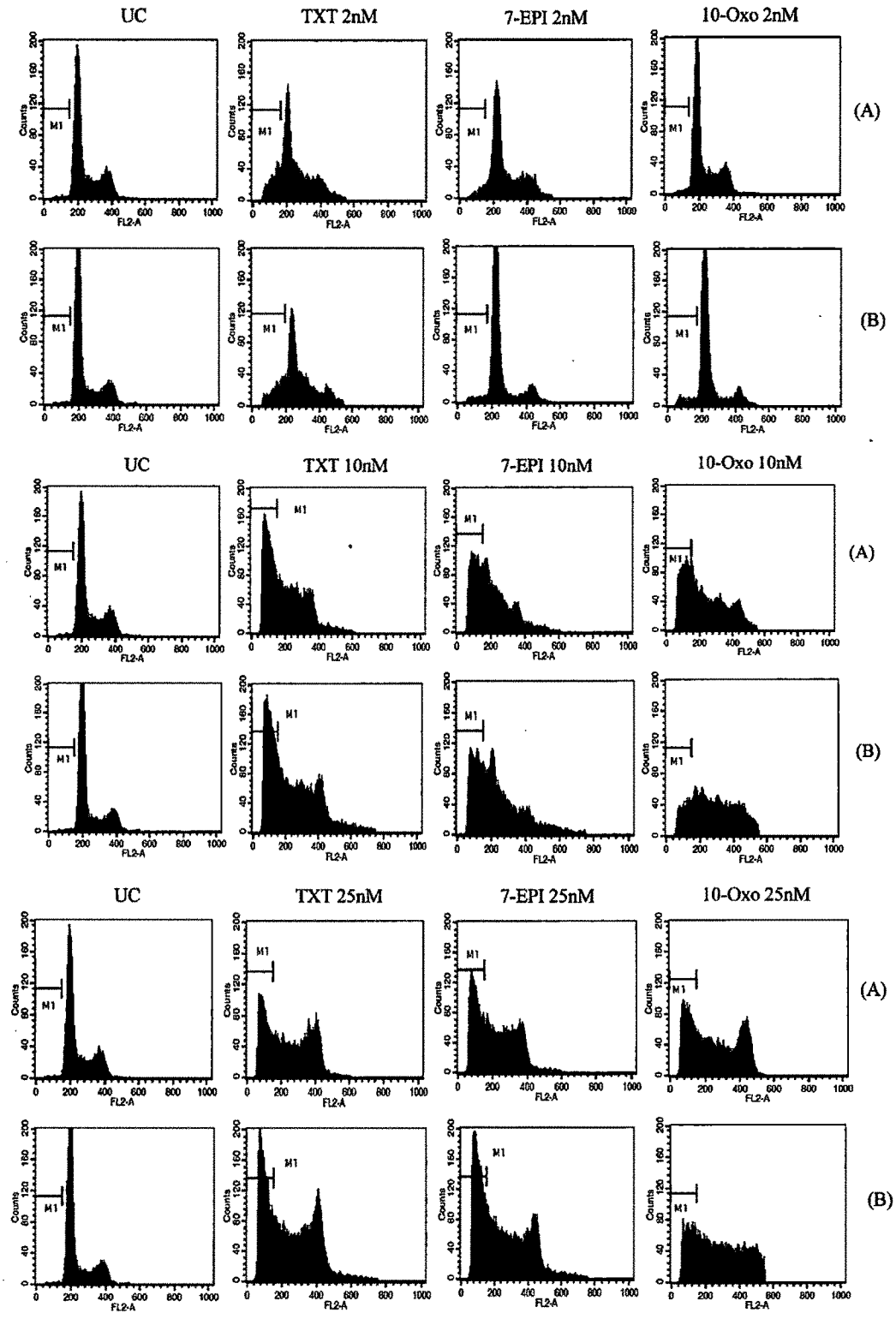


Figure 10.11. A549 cell apoptosis after treatment with Taxotere (TXT), 7-epidocetaxel (7-EPI), and 10-oxo-7-epidocetaxel (10-oxo) at 2nM, 10nM, and 25nM concentrations for 24 hr (A) and 42hr (B).

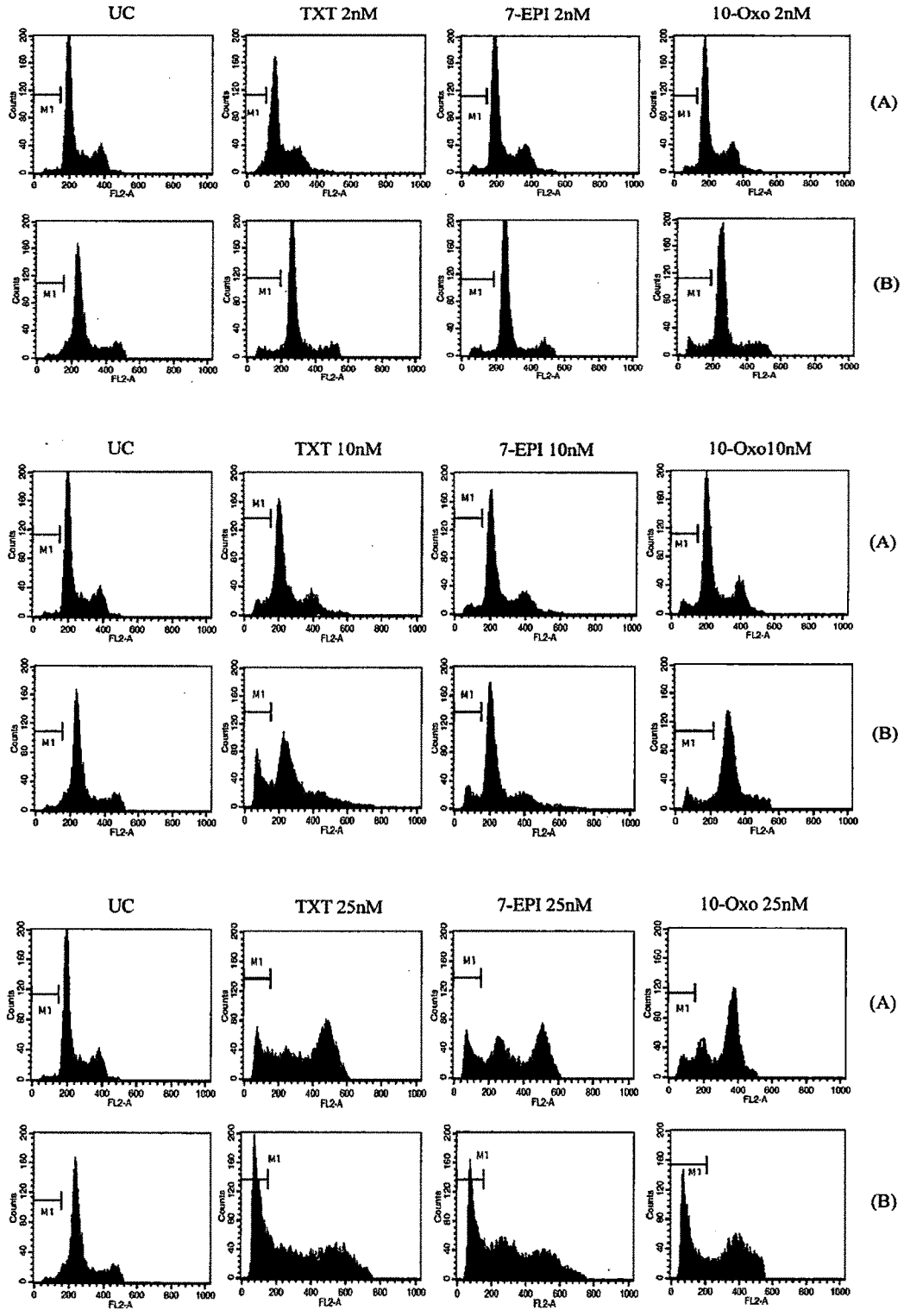


Figure 10.12. B16F10 cell apoptosis after treatment with Taxotere (TXT), 7-epidocetaxel (7-EPI) and 10-oxo-7-epidocetaxel (10-oxo) at 2nM, 10nM, and 25nM concentrations for 24 hr (A) and 42hr (B).

Table 10.2. The % A549 and B16F10 cell apoptosis after treatment with 1mL of 2nM, 10nM, and 25nM of Taxotere, 7-epidocetaxel, and 10-oxo-7-epidocetaxel for a period of 24hr and 48hr

Drug	% Apoptosis of A549 cells		% Apoptosis of B16F10	
	24 hr	**48hr**	**24 hr**	**48hr**
UC	0.485±0.12	0.43±0	1.03±0.09	1.13±0.16
TXT- 2nM	18.55±0.5	16.7±4.65	3.66±0.05	6.33±0.9
TXT-10nM	42.57±1.89	40.76±2.07	11.175±1.61	20.88±1.09
TXT-25nM	34.08±0.64	36.11±0.31	20.75±0.95	38.18±1.3
7-EPI- 2nM	3.81±0.84	2.11±1.41	2.36±0.02	4.29±0.8
7-EPI-10nM	39.18±1.52	35.78±1.61	4.95±1.02	10.56±0.62
7-EPI-25nM	37.36±1.6	38.2±0.7	18.63±1.85	33.5±1.18
10-Oxo-2nM	1.84±0.47	1.265±0.02	3±0.34	7.84±2.08
10-Oxo-10nM	34.82±0.78	19.04±1.11	5.2±1.69	12±0.65
10-Oxo-25nM	30.32±2.56	27.59±2.87	12.98±1.2	39.02±5.33

Values are Mean±SD, n=3. TXT: Taxotere; 7-EPI: 7-epidcoetaxel; 10-Oxo: 10-oxo-7-epidocetaxel. All formulations, except 7-epidocetaxel and 10-oxo-7-epidocetaxel at 2nM concentration, tested caused significant A549 cell apoptosis ($p<0.0001$) at both 24hr and 48hr treatment as compared to untreated cells. The Taxotere caused significant A549 cell apoptosis at 10nM as compared to 10-oxo-7-epidocetaxel ($p<0.0001$) after 24hr treatment and 48hr treatment. The Taxotere caused significant A549 cell apoptosis at 25nM as compared to 10-oxo-7-epidocetaxel ($p<0.01$, $p=0.002$) after 48hr treatment. Similarly, all formulations, except Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel at 2nM concentration, tested caused significant B16F10 cell apoptosis at 24hr treatment ($p<0.05$) as compared to untreated cells. After 48hr treatment all formulations, except Taxotere and 7-epidocetaxel at 2nM concentration, caused significant apoptosis as compared to untreated cells ($p<0.0001$; 10-oxo-7-epidocetaxel at 2nM: $p<0.05$). The Taxotere caused significant B16F10 cell apoptosis at 10nM and 25nM as compared to 10-oxo-7-epidocetaxel ($p<0.0001$) after 24hr treatment. After 48hr treatment Taxotere caused significant B16F10 cell apoptosis at 10nM as compared to 10-oxo-7-epidocetaxel ($p<0.01$, $p=0.001$). The Taxotere caused significant B16F10 cell apoptosis at 10nM as compared to 7-epidocetaxel after 24hr and 48hr of treatment ($p<0.001$).

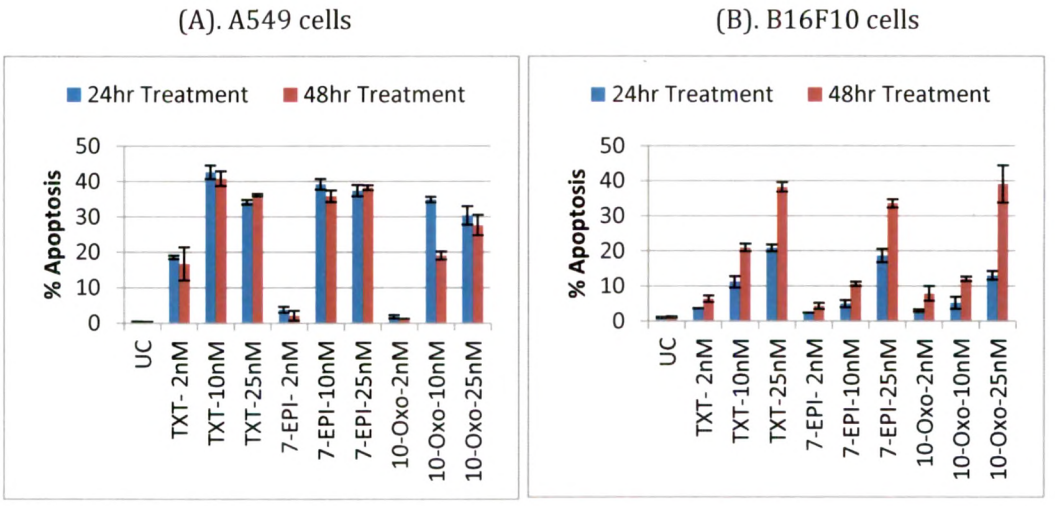


Figure 10.13. The % A549 (A) and B16F10 (B) cell apoptosis after treatment with 1mL of 2nM, 10nM, and 25nM of Taxotere (TXT), 7-epidocetaxel (7-EPI) and 10-oxo-7-epidocetaxel (10-oxo) for a period of 24hr and 48hr

10.11.6. Cell cycle analysis

To determine the effect of docetaxel (Taxotere) and its impurities, 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel on cell cycle, the A549 and B16F10 cells were treated with 2mL of 2nM, 10nM and 25nM solution of docetaxel and its impurities for a period of 24hr and 48hr. The drug treated cells were then acquired on FACS Calibur and analysed using ModFit LT V2.0 (PMac) software to determine the percentage of A549 (Figure 10.14a-c) and B16F10 (Figure 10.15a-c) cells present in G0-G1, S and G2-M phase of cell cycle.

The treatment of A549 cells with 2nM concentration the Taxotere for 24hr caused about 5.12% increased cell accumulation in G2-M phase as compared to untreated cells, whereas 7EDTX and 10-oxo-7-epidocetaxel caused no effect. The 48 hr treatment with Taxotere caused about 10.37% increased accumulation of cells at S phase as compared to untreated cells, whereas 7EDTX and 10-oxo-7-epidocetaxel caused no effect even after 48hr treatment indicating they less effective on A549 cell cycle at 2nM concentration as compared to Taxotere (Table 10.3a and Figure 10.16a).

The about 31.68%, 25.37% and 7.96% increased accumulation of A549 cells at S phase and 14.62%, 23.43% and 20.06% increased accumulation of A549 cells at G2-M phase as compared to untreated cells was observed after treatment with 10nM concentration of Taxotere, 7EDTX and 10-oxo-7-epidocetaxel, respectively for 24hr. Whereas, the 48hr treatment caused 42.22%, 35.62% and 37.84% increased accumulation in S phase with 10nM of Taxotere and 7EDT and 10-oxo-7-epidocetaxel, respectively. Also, we observed about 20.06% and 12.77% increased accumulation of A549 cells at G2-M phase after

48hr treatment with Taxotere and 10-oxo-7-epidocetaxel, respectively but no change at G2-M phase was observed with 7EDTX treatment (Table 10.3b and Figure 10.16b).
Similarly, the about 6.31%, 30.9% and 30.08% increased accumulation of A549 cells at S phase and 39.74%, 27.69% and 20.67% increased accumulation at G2-M phase as compared to untreated cells was observed after treatment with 25nM concentration of Taxotere, 7EDTX and 10-oxo-7-epidocetaxel, respectively for 24hr. Whereas, the 48hr treatment with Taxotere and 7EDT and 10-oxo-7-epidocetaxel caused about 34.34%, 30.9% and 37.08% increased accumulation of A549 cells in S phase, respectively and 33.25%, 27.69% and 20.67% increased accumulation of A549 cells at G2-M phase, respectively as compared to untreated cells (Table 10.3c and Figure 10.16c).
At lower concentration of 2nM, the impurities showed no changes in the S phase and G2-M phase after both 24hr and 48hr treatment, whereas Taxotere showed slight increase in G2-M phase (5.12%) after 24hr treatment and S phase (10.37%) after 48hr treatment. This indicates Taxotere is more effective even at low concentration than docetaxel impurities tested.
At 10nM concentration and 24hr treatment, Taxotere and 7DTX caused about 2.1 fold and 5.3 fold increased accumulation of A549 cells at S phase, respectively as compared to G2-M phase, whereas 10-oxo-7-epidocetaxel caused 2.94 fold increased accumulation of A549 cells at G2-M phase as compared to S phase. Similarly after 48hr treatment the increased accumulation of A549 cells in S phase as compared to G2-M phase was observed with Taxotere (2.1 fold), 7EDTX (no change in G2-M phase) and 10-oxo-7-epidocetaxel (2.96 fold).
At 25nM concentration and 24hr treatment, the Taxotere caused 6.29 fold increased accumulation of A549 cells at G2-M phase as compared to S phase, whereas 10-oxo-7-epidocetaxel caused 1.79 fold increased accumulation of A549 cells at S phase as compared to G2-M phase. Overall, we observed that the docetaxel and its impurities blocked the A549 cell cycle at both S phase and G2-M phase based on their concentration and treatment time. At 24hr treatment with 10nM, we observed that the Taxotere caused more accumulation of cells at S phase, whereas the 10-oxo-docetaxel blocked more at G2-M phase and vice versa at 24hr treatment with 25nM concentration.
The treatment of B16F10 cells with 2nM concentration of 7EDTX and 10-oxo-7-epidocetaxel for 24hr caused about 3.95% and 5.66% increased S phase, respectively as compared to untreated cells, whereas Taxotere caused no changes. The 48 hr treatment with Taxotere, 7EDTX and 10-oxo-7-epidocetaxel caused about 3.56%, 3.36% and 2.47% increased accumulation of cells at S phase, respectively as compared to untreated cells, whereas no changes were observed at G2-M phase (Table 10.4a and Figure 10.17a).
The about 3.76% increased accumulation of B16F10 cells at G2-M phase as compared to untreated cells was observed after treatment with 10nM concentration of 10-oxo-7-epidocetaxel, whereas no changes were observed with Taxotere and 7EDTX treatment. We observed about 31.04% increased accumulation of B16F10 cells at S phase and 5.9%

increased accumulation of B16F10 cells at G2-M phase as compared to untreated cells after 48hr treatment with Taxotere and 10-oxo-7-epidocetaxel, respectively but no changes were observed with 7EDTX treatment (Table 10.4b and Figure 10.17b).

The about 57.02%, 44.31% and 42.47% increased accumulation of B16F10 cells at G2-M phase as compared to untreated cells was observed after 24hr treatment with 25nM concentration of Taxotere, 7EDTX and 10-oxo-7-epidocetaxel, respectively. Similarly, the 48hr treatment with Taxotere and 7EDT and 10-oxo-7-epidocetaxel caused 63.52%, 40.76% and 39.29% increased accumulation of B16F10 cells at G2-M phase, respectively as compared to untreated cells. The 10-oxo-7-epidocetaxel also caused 13.14% increased accumulation of B16F10 cells at S phase after 48hr treatment (Table 10.4c and Figure 10.17c).

The results indicate that the B16F10 cells are less sensitive at both 2nM and 10nM as compared to A549 cells. Although, no accumulation of cells at S phase was observed with Taxotere and 7EDTX at all concentrations and treatment times as compared to A549 cells, the 10-oxo-7-epidocetaxel caused about 13.14% increased accumulation of B16F10 cells at S phase after 48hr treatment with 25nM concentration.

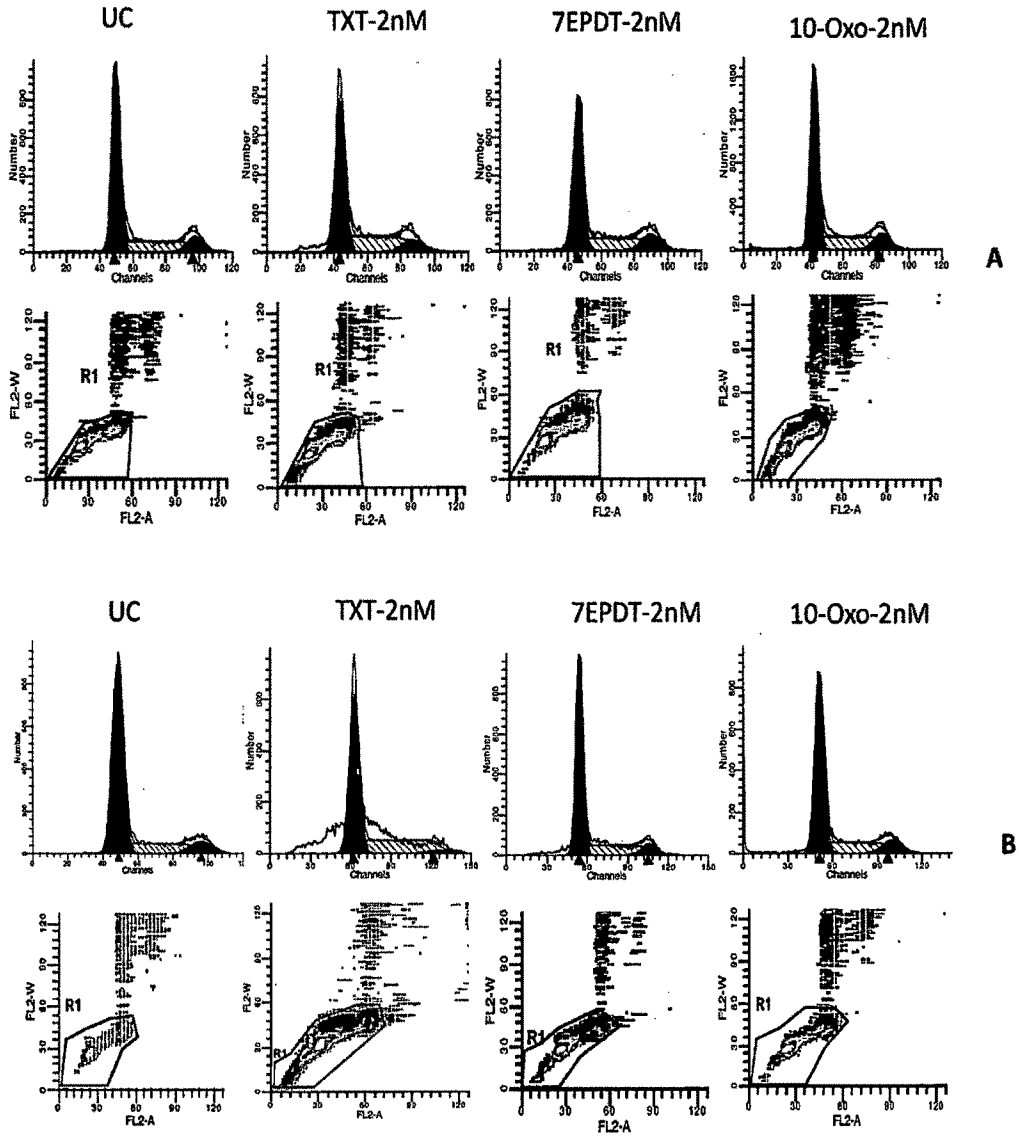


Figure 10.14(a). The FACS analysis of % of A549 cells in G0-G1, S and G2-M phase after treatment with 2nM solution of Taxotere (TXT), 7-epidocetaxel (7-EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of (A): 24hr and (B): 48hr

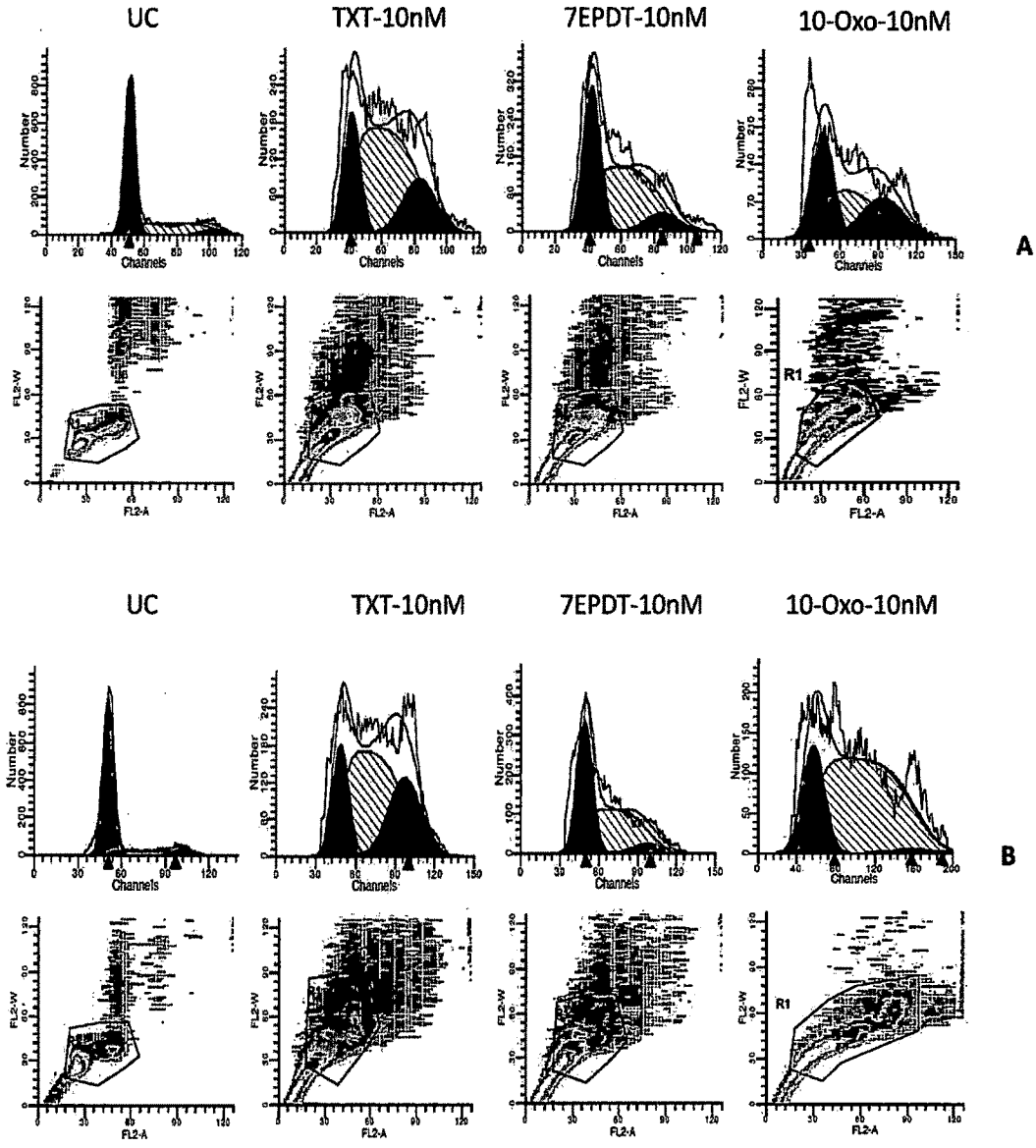


Figure 10.14(b). The FACS analysis of % of A549 cells in G0-G1, S and G2-M phase after treatment with 10nM solution of Taxotere (TXT), 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of (A): 24hr and (B): 48hr

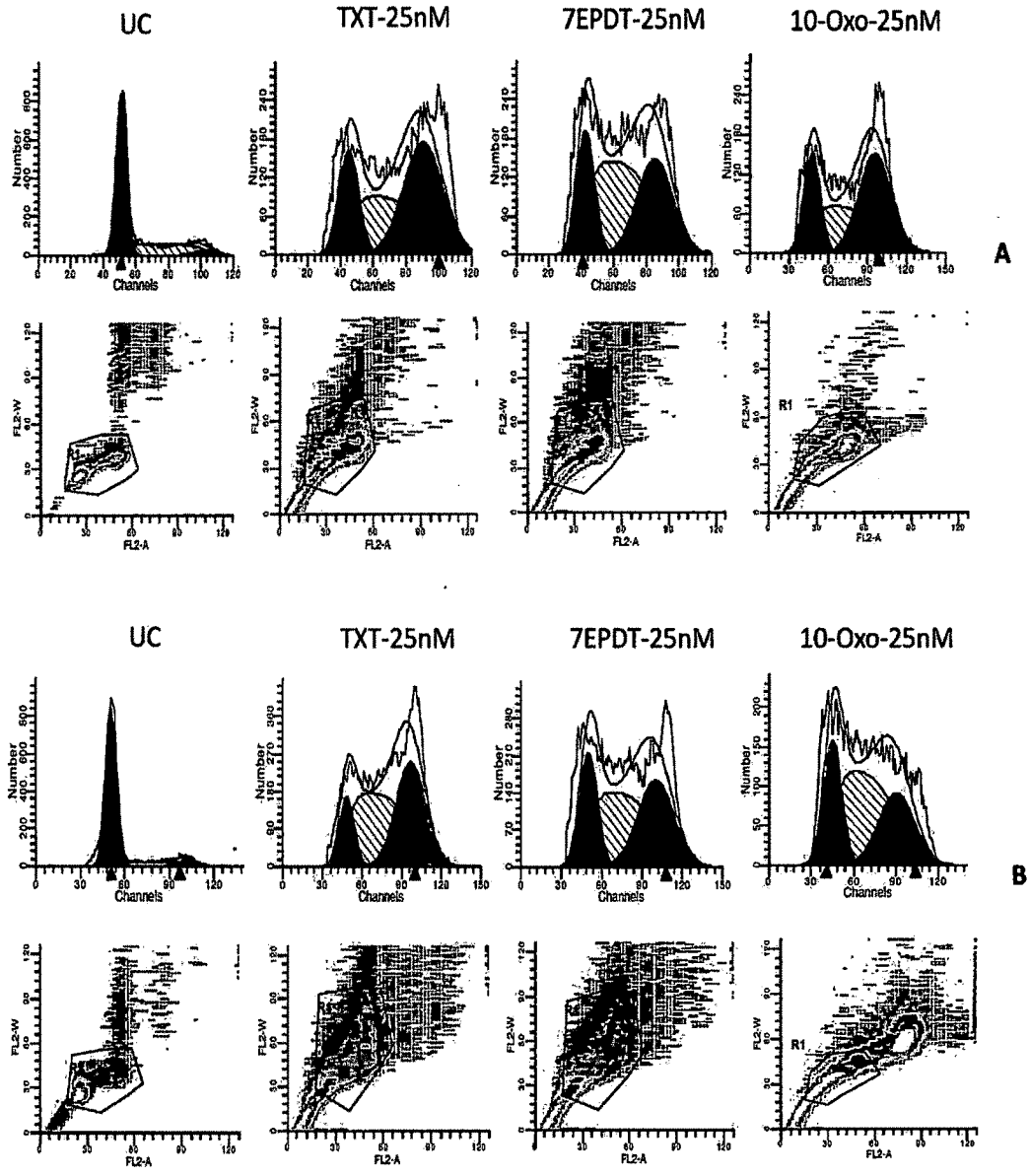


Figure 10.14(c). The FACS analysis of % of A549 cells in G0-G1, S and G2-M phase after treatment with 25nM solution of Taxotere (TXT), 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of (A): 24hr and (B): 48hr

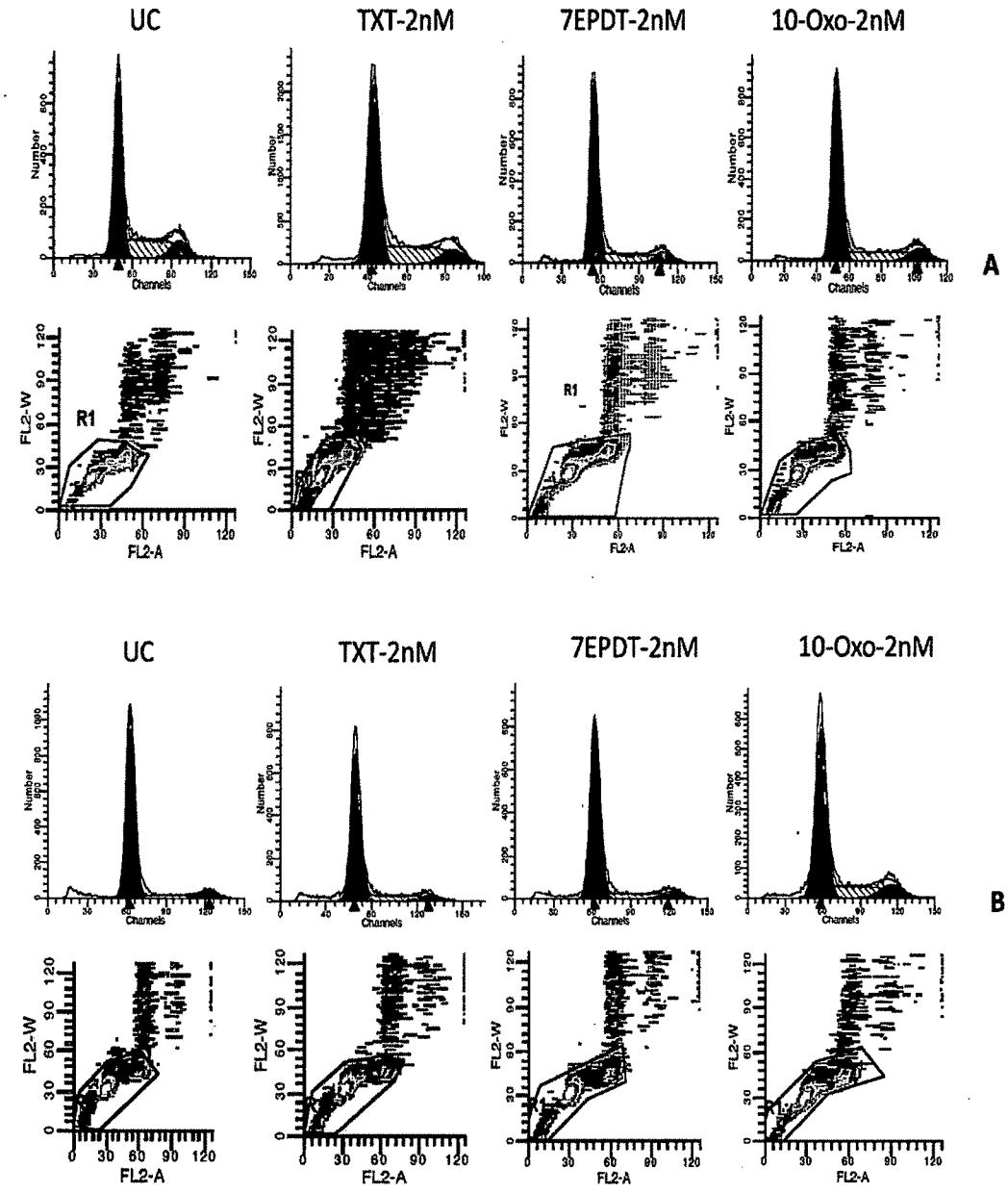


Figure 10.15(a). The FACS analysis of % of B16F10 cells in G0-G1, S and G2-M phase after treatment with 2nM solution of Taxotere (TXT), 7-epidocetaxel (7EDTX), and 10-oxo-7-epidocetaxel (10-oxo) for a period of (A): 24hr and (B): 48hr

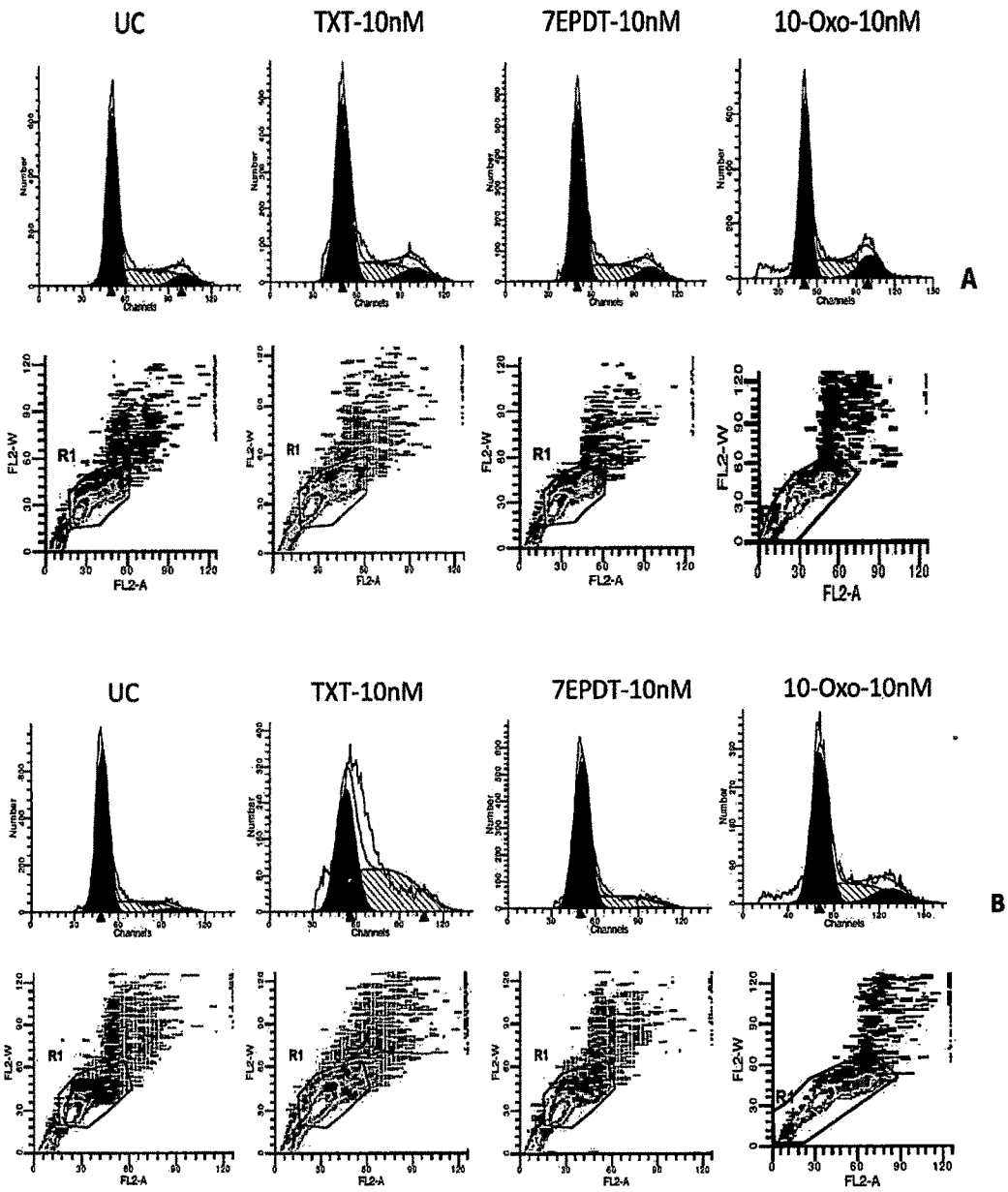


Figure 10.15(b). The FACS analysis of % of B16F10 cells in G0-G1, S and G2-M phase after treatment with 10nM solution of Taxotere (TXT), 7-epidocetaxel (7EDTX), and 10-oxo-7-epidocetaxel (10-oxo) for a period of (A): 24hr and (B): 48hr

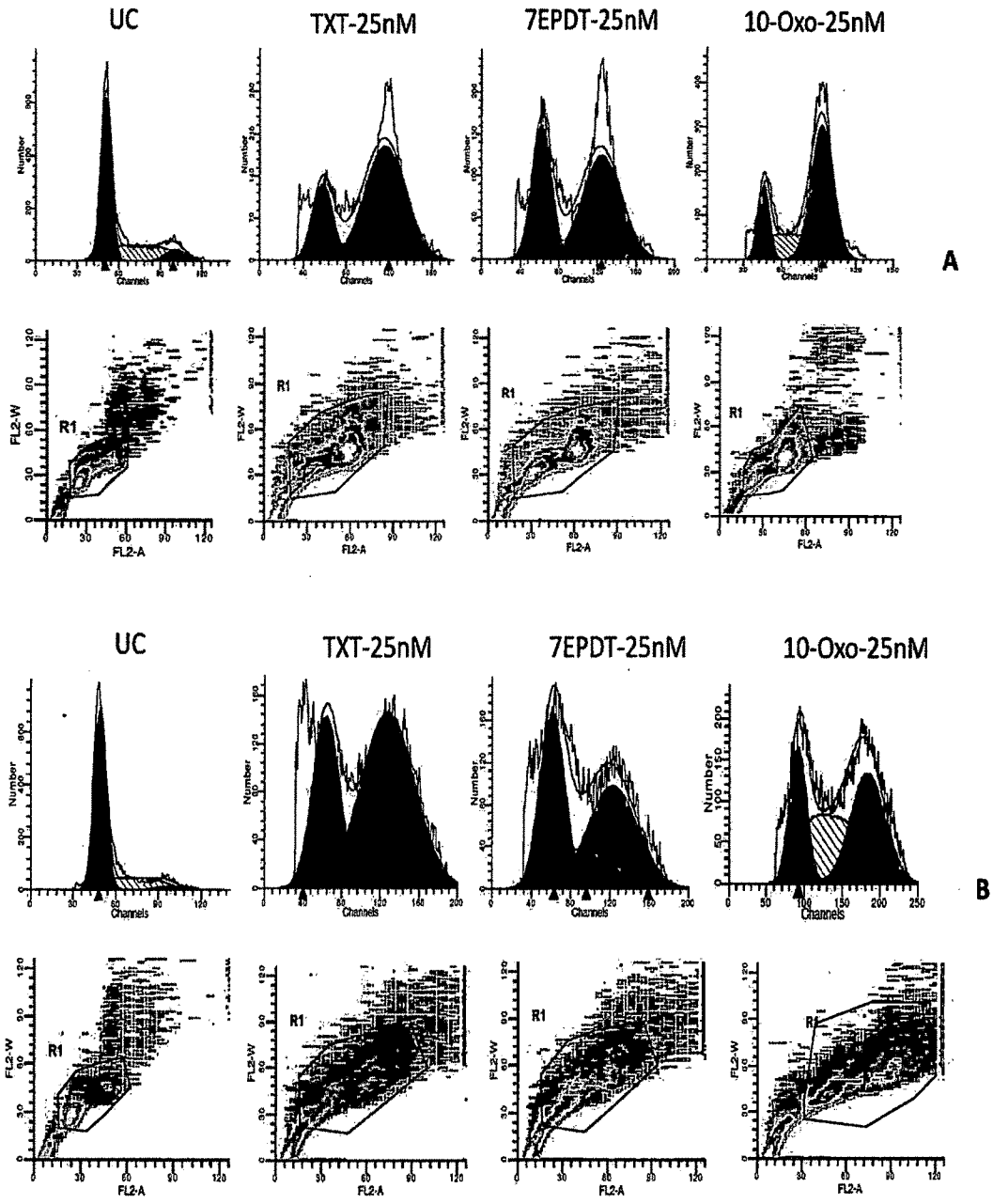


Figure 10.15(c). The FACS analysis of % of B16F10 cells in G0-G1, S and G2-M phase after treatment with 25nM solution of Taxotere (TXT), 7-epidocetaxel (7EDTX), and 10-oxo-7-epidocetaxel (10-oxo) for a period of (A): 24hr and (B): 48hr

Table 10.3(a). The % of A549 cells in G0-G1, S and G2-M phase after treatment with 2nM solution of Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel for a period of 24hr and 48hr.

Cell cycle phase	24hr treatment			
	UC	TXT	7EDX	10-Oxo
G0-G1	60.14±1.13	54.57±1.35	57.32±1.9	57.45±0.47
S	29.34±0.62	29.78±6.56	31.66±3.4	32.25±1.04
G2-M	10.52±0.71	15.64±8.91	10.98±2.12	10.29±0.76
	48hr treatment			
G0-G1	76.17±6.9	70.53±13.8	74.69±8.1	74.42±8.7
S	16.71±5.4	27.08±14.11	16.31±10.2	18.78±6.01
G2-M	7.09±1.5	2.38±0.24	8.99±2.19	6.8±2.7

Values are Mean±SD, n=3. TXT: Taxotere; 7EDTX: 7-epidocetaxel; 10-Oxo: 10-oxo-7-epidocetaxel

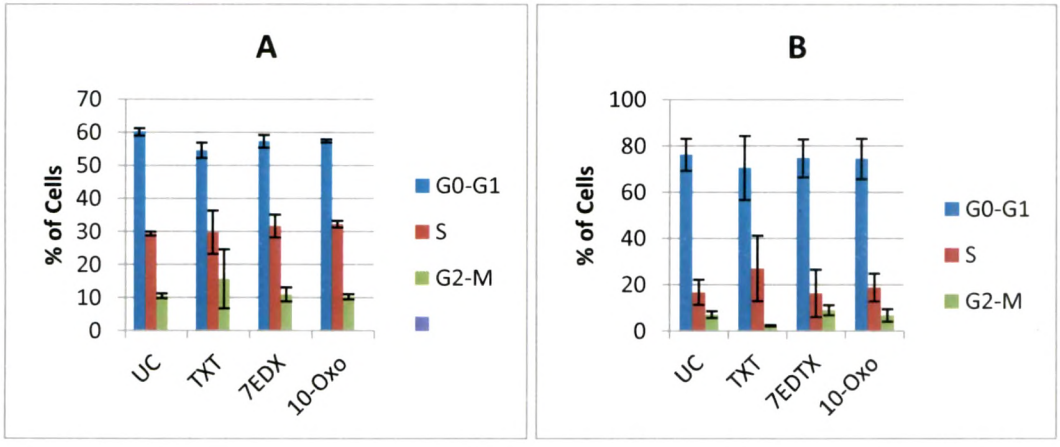


Figure 10.16(a). The % of A549 cells in G0-G1, S and G2-M phase after treatment with 2nM solution of Taxotere (TXT), 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of (A): 24hr and (B): 48hr.

Table 10.3(b). The % of A549 cells in G0-G1, S and G2-M phase after treatment with 10nM solution of Taxotere (TXT), 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of 24hr and 48hr.

Cell cycle phase	24hr treatment			
	UC	TXT	7EDX	10-Oxo
G0-G1	66.73±1.53	20.89±2.3	36.61±1.4	35.34±2.6
S	28.94±2.6	60.62±3.56	54.31±3.8	36.90±1.5
G2-M	4.32±1.7	18.49±4.9	9.08±2.2	27.75±2.2
	48hr treatment			
G0-G1	80.73±4.9	18.45±6.8	44.53±6.2	30.125±9.7
S	13.36±2.4	55.58±12.1	48.98±4.2	51.2±13
G2-M	5.91±1.5	25.97±2.2	6.49±2.4	18.68±12.7

Values are Mean±SD, n=3. TXT: Taxotere; 7EDTX: 7-epidocetaxel; 10-Oxo: 10-oxo-7-epidocetaxel

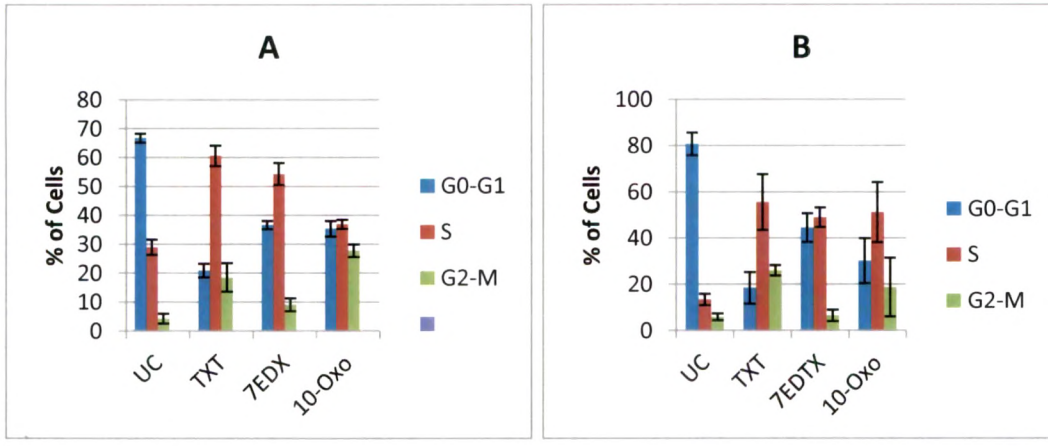


Figure 10.16(b). The % of A549 cells in G0-G1, S and G2-M phase after treatment with 10nM solution of Taxotere (TXT), 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of (A): 24hr and (B): 48hr

Table 10.3(c). The % of A549 cells in G0-G1, S and G2-M phase after treatment with 25nM solution of Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel for a period of 24hr and 48hr.

Cell cycle phase	24hr treatment			
	UC	TXT	7EDX	10-Oxo
G0-G1	66.73±1.1	20.69±4.3	20.29±2.1	28.40±5.2
S	28.94±2.62	35.25±5.6	48.34±3.9	27.06±5.7
G2-M	4.32±1.71	44.06±7.1	31.37±1.9	44.52±1.6
	48hr treatment			
G0-G1	80.73±5.9	13.14±9.8	22.14±5.1	22.99±6.1
S	13.36±4.4	47.7±6.1	44.26±8.2	50.44±4
G2-M	5.91±2.9	39.16±3.2	33.6±3.1	26.58±4.7

Values are Mean±SD, n=3. TXT: Taxotere; 7EDTX: 7-epidocetaxel; 10-Oxo: 10-oxo-7-epidocetaxel

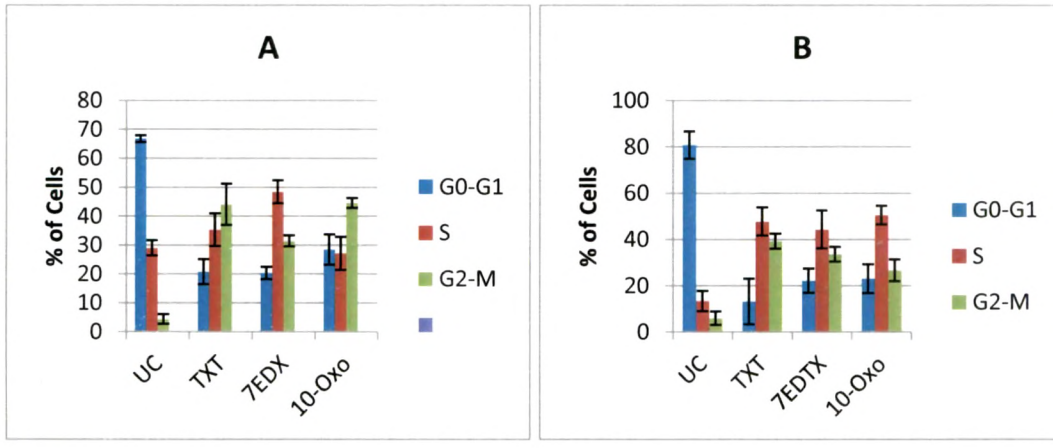


Figure 10.16(c). The % of A549 cells in G0-G1, S and G2-M phase after treatment with 25nM solution of (TXT), 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of 24hr and 48hr.

Table 10.4(a). The % of B16F10 cells in G0-G1, S and G2-M phase after treatment with 2nM solution of Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel for a period of 24hr and 48hr.

Cell cycle phase	24hr treatment			
	UC	TXT	7EDTX	10-Oxo
G0-G1	63.54±12.6	63.71±5.7	61.27±6.5	59.84±6.4
S	24.46±10.4	23.88±7.2	28.41±4.5	30.06±5.4
G2-M	11.99±2.2	12.4±5.5	10.31±2.4	10.1±1.15
	48hr treatment			
G0-G1	76.27±7.7	72.92±6.5	71.86±9.1	72.7±8.8
S	17.04±6.63	20.6±4.1	20.4±7.3	19.51±5.5
G2-M	6.69±1.1	6.48±2.3	7.73±1.8	7.79±3.3

Values are Mean±SD, n=3. TXT: Taxotere; 7EDTX: 7-epidocetaxel; 10-Oxo: 10-oxo-7-epidocetaxel

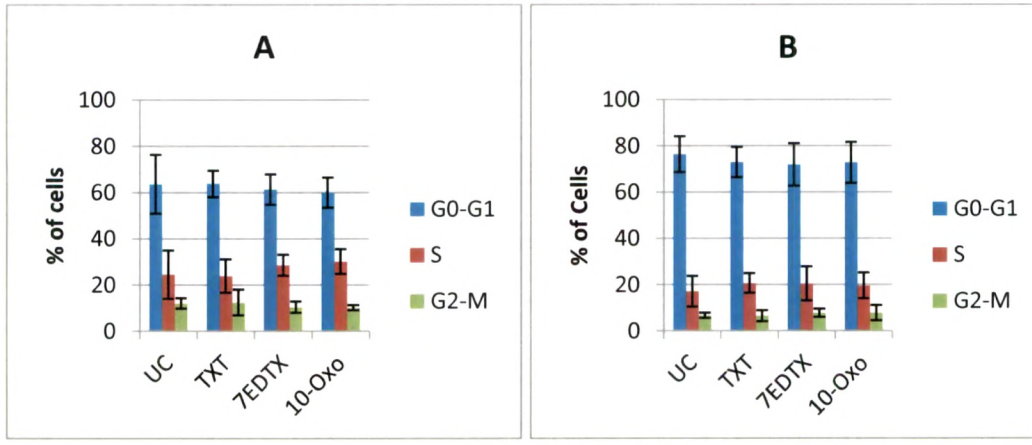


Figure 10.17(a). The % of B16F10 cells in G0-G1, S and G2-M phase after treatment with 2nM solution of (TXT), 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of 24hr and 48hr.

Table 10.4(b). The % of B16F10 cells in G0-G1, S and G2-M phase after treatment with 10nM solution of Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel for a period of 24hr and 48hr.

Cell cycle phase	24hr treatment			
	UC	TXT	7EDTX	10-Oxo
G0-G1	61.46±10.6	63.19±5.7	62.28±3.1	58.76±5.6
S	29.76±8.4	27.38±7.2	27.58±2.4	28.69±4.1
G2-M	8.78±2.2	9.43±5.5	10.14±1.4	12.54±2.4
	48 hr treatment			
G0-G1	74.96±6.7	46.21±6.5	74.28±6.9	66.43±7.7
S	21.55±4.6	52.59±4.1	23.95±5.2	24.17±4.7
G2-M	3.49±1.5	1.2±0.8	1.77±1.8	9.39±3

Values are Mean±SD, n=3. TXT: Taxotere; 7EDTX: 7-epidocetaxel; 10-Oxo: 10-oxo-7-epidocetaxel

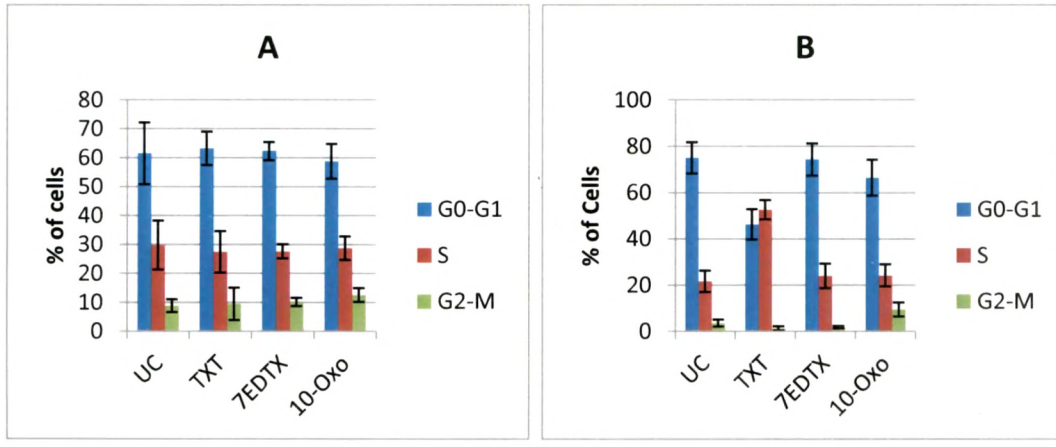


Figure 10.17(b). The % of B16F10 cells in G0-G1, S and G2-M phase after treatment with 10nM solution of (TXT), 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of 24hr and 48hr.

Table 10.4(c). The % of B16F10 cells in G0-G1, S and G2-M phase after treatment with 25nM solution of Taxotere, 7-epidocetaxel and 10-oxo-7-epidocetaxel for a period of 24hr and 48hr.

Cell cycle phase	24hr treatment			
	UC	TXT	7EDTX	10-Oxo
G0-G1	61.46±11.7	22.05±8.7	34.44±7.5	24.28±16.7
S	29.76±9.9	12.16±7.2	12.46±5.5	24.29±2.5
G2-M	8.78±1.8	65.8±9.5	53.09±8.4	51.25±14.6
	48hr treatment			
G0-G1	74.96±11.3	32.99±7.5	37.84±5.1	22.52±1.1
S	21.55±9.6	2.6±1.1	17.91±1.3	34.69±6.1
G2-M	3.49±1.6	67.01±9.3	44.25±4.8	42.78±7.2

Values are Mean±SD, n=3. TXT: Taxotere; 7EDTX: 7-epidocetaxel; 10-Oxo: 10-oxo-7-epidocetaxel

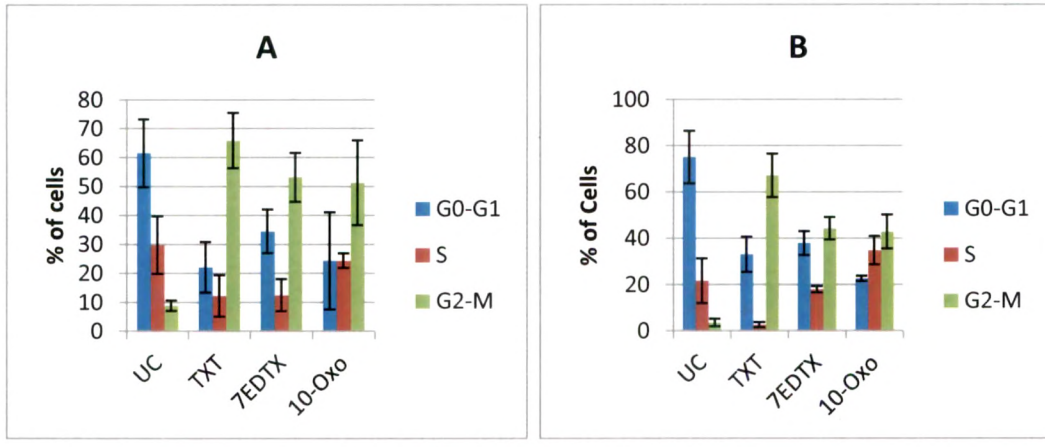


Figure 10.17(c). The % of B16F1o cells in G0-G1, S and G2-M phase after treatment with 25nM solution of (TXT), 7-epidocetaxel (7EDTX) and 10-oxo-7-epidocetaxel (10-oxo) for a period of 24hr and 48hr.

10.11.7. Acute toxicity study

An *in-vivo* B16F10 melanoma lung metastasis mice model (Hwang et al., 2001; Gautam et al., 2002; Chen et al., 2009) was used to compare the single *i.v.* dose acute toxicity of Taxotere for injection alone and with 10% 7-epidocetaxel and 10-oxo-7-epidocetaxel impurities (Dora et al., 2006; Chen et al., 2010). The weight measurements of the mice were performed during the experiments to evaluate the toxicity of Taxotere for injection and Taxotere containing 10% docetaxel impurities separately (Table 10.5 and Figure 10.18). At the end of the experiment, the significant weight loss was observed with Taxotere alone and Taxotere with 10% 7-epidocetaxel treated groups as compared to untreated control group.

The control group shows 1.9, 4.3, 7.2, 8.6 and 6.3% mean group weight loss at 2, 4, 6, 8, and 10th day after PBS administration, respectively. The Taxotere injected group shows 3.9, 7.4, 10.4, 17.2, and 25.5% mean group weight loss at 2, 4, 6, 8, and 10th day after Taxotere administration at a dose of 40mg/kg. As compared to control group about 2.09, 1.73, 1.44, 1.98, and 4.05 fold increase in mean group weight loss was observed with Taxotere treated group at 2, 4, 6, 8, and 10th day, respectively. This increased mean group weight loss can be correlated to the toxicity of Taxotere injection. The group treated with Taxotere containing 10% 7-epidocetaxel shows 6.2, 11.1, 14.1, 19.3 and 24.1% mean group weight loss at 2, 4, 6, 8, and 10th day of dose administration, respectively. As compared to control group the Taxotere containing 10% 7-epidocetaxel treated group showed about 3.27, 2.6, 1.96, 2.22, and 3.82 fold increase in mean group weight loss at 2, 4, 6, 8, and 10th day of drug administration. The inclusion of 10% 7-epidocetaxel in Taxotere injection results in 1.56, 1.5, 1.35, and 1.12 fold increase in mean group weight loss as compared to Taxotere containing no 7-epidocetaxel at 2, 4, 6 and 8 day of drug administration, respectively. This extra loss of body weight indicates the more toxicity of 7-epimer as compared to docetaxel. Czejka et al. (Czejka et al., 2010) reported, first time, the quantification of 7-epidocetaxel in blood and urine of chemotherapy patients (Taxotere). Therefore we can say that the total toxicity that occurs during chemotherapy with Taxotere is because of both docetaxel and 7-epimer formed in blood after Taxotere infusion. The *in vivo* acute toxicity study revealed the more toxicity after inclusion of 10% 7-epimer to Taxotere as compared to Taxotere alone. Therefore, it is very clear that the prevention of conversion of docetaxel into 7-epimer *in vivo* can enhance the therapeutic effectiveness of Taxotere with decreased toxicity. Bornique and Lemarie proved the role of 7-epimer in inducing human cytochrome P450 1B1 (hCYP1B1) which is present in various human tumors and is postulated to be responsible for the development of resistance of tumor cells toward chemotherapeutic agents, including docetaxel (Bornique and Lemarie, 2002). Thus controlling the formation of 7-epimer *in vivo* will also reduce the chance of tumor resistance to chemotherapy. As per our stability results the docetaxel remain stable at acidic pH, and also the tumor environment is acidic in nature (Tannock and Rotin, 1989; Mahoney et al., 2003). Thus, the docetaxel in tumor tissue would remain more stable (no

conversion to 7-epimer) than that in circulation (blood pH of 7.4). Thus pharmaceutical scientist must look towards an alternative drug delivery systems (like liposomes in our study) which control the release the docetaxel in circulation (we can expect *in vivo* controlled release from our *in vitro* DTX release data) and passively accumulate the docetaxel to tumor tissue and the released docetaxel at acidic tumor tissue might less/never converted to 7-epimer and thus might show better therapeutic effect with minimum toxicity and minimum chance of tumor resistance to chemotherapy. Further studies are needed to ascertain these facts.

We also tested the toxicity of Taxotere containing 10% 10-oxo-7-epidocetaxel (another docetaxel impurity) at 40mg/kg dose. At 2nd day of dose administration we observed negligible mean body weight loss (0.102%) as compared to groups treated with Taxotere, Taxotere containing 7-epimer and even control group. At 4, 6, 8, and 10th day this group shows 8.5, 9.4, 14.5, and 15.1% mean body weight loss, respectively. This group showed about 1.99, 1.3, 2.19 and 2.39 fold increase in mean group weight loss as compared to control group at 4, 6, 8, and 10th day, respectively. When we compared the toxicity of this group with Taxotere treated group, we observed almost similar toxicity at 4th and 6th day, but at 8 and 10th day about 2.68% and 10.4% decreased body weight loss was observed as compared to Taxotere treated group. Surprisingly, we observed the less toxicity with Taxotere containing 10% 10-oxo-7-epidocetaxel as compared to Taxotere alone. The inclusion of 10% 10-oxo-7-epidocetaxel surprisingly decreased the total toxicity of Taxotere.

The Taxotere with 10% 10-oxo-7-epi-docetaxel treated group showed about 6.1, 2.6, 4.7, 4.7, and 9% decreased average body weight loss as compared to Taxotere containing 10% 7-epimer treated group at 2, 4, 6, 8, and 10th day of dose administration, respectively.

On 19th day of experiment the one animal from the control group and one animal from Taxotere with 10% 7-epimer treated group were found dead. On 20th day the animals were sacrificed and lungs were excised and photographed (Figure 10.19). The lungs of the control group, Taxotere treated group and Taxotere with 10% 7-epimer treated group were found completely black and surface metastatic colonies were remain uncountable (Figure 10.19A, B and C). Therefore no therapeutic effect (decreased colony formation) was observed with Taxotere and Taxotere with 10% 7-epimer treated groups at 40mg/kg single dose as compared to untreated control group. Surprisingly, we observed decreased surface metastatic colony formation with Taxotere containing 10% 10-oxo-7-epidocetaxel treated group (Figure 10.19D) at the same single dose (40mg/kg) as compared to all other groups. The lung sections were fixed, stained with hematoxylin-eosin and observed under phase contrast microscope (Axio Imager. Z1 upright microscope, Germany). The images of control, Taxotere alone and Taxotere with 10% 7-epidocetaxel treated group showed large and fused nodules as compared to 10-oxo-7-epidocetaxel treated group which showed small and distinct nodules (Figure 10.20).

In conclusion, we observed surprisingly the decreased % average body weight loss and decreased number of surface colonies with Taxotere containing 10% 10-oxo-7-epidocetaxel treated group as compared to Taxotere treated group and Taxotere containing 10% 7-epimer treated group. Therefore, the further evaluation of the therapeutic effectiveness of the docetaxel impurity, 10-oxo-7-epidocetaxel, alone in mice pulmonary metastatic model and/or in other tumors in comparison to approved Taxotere and other docetaxel formulations is necessary to confirm our results.

Table 10.5. The % group weights after administration of Taxotere alone and Taxotere with 10% impurities separately

Day	UC	TXT	TXT with 10% 7-EPI	TXT with 10% 10-Oxo
0*	100±7.25	100±11.91	100±8.79	100±7.63
2	98.09±6.03	96.01±12.7	93.75±7.09	99.89±10.11
4	95.69±5.36	92.53±8.84	88.8±9.53	91.4±6.98
6	92.76±9.88	89.55±11.05	85.8±7.5	90.53±6.89
8	91.3±6.7	82.73±9.33	80.63±7.98	85.42±7.96
10	93.66±5.1	74.76±6.35	75.9±5.5	84.88±7.6

0*: Day when the drugs are administered, Values represented are Mean ±SD.
TXT: Taxotere; 7-EPI: 7-epidcoetaxel; 10-Oxo: 10-oxo-7-epidocetaxel

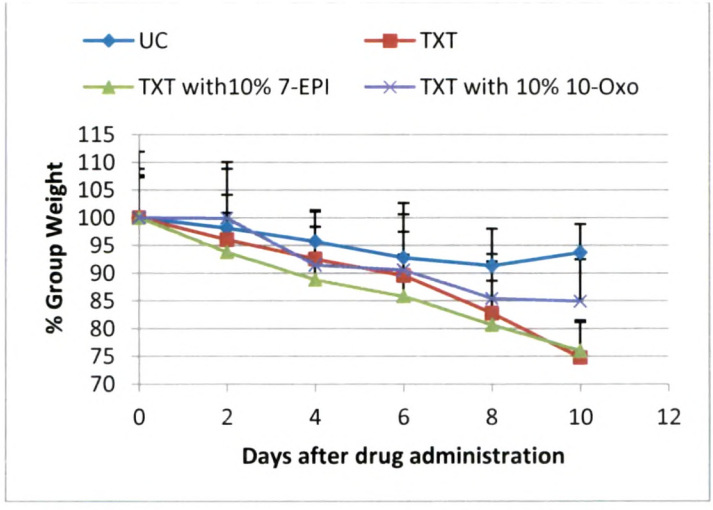


Figure 10.18. The % group weights after administration of Taxotere alone and Taxotere with 10% impurities separately. The group weight of mice treated with Taxotere ($p<0.05$, $p=0.034$) and Taxotere containing 10% 7-epidocetaxel ($p<0.05$, $p=0.048$) was decreased significantly at 10th day after dose administration as compared to control group.

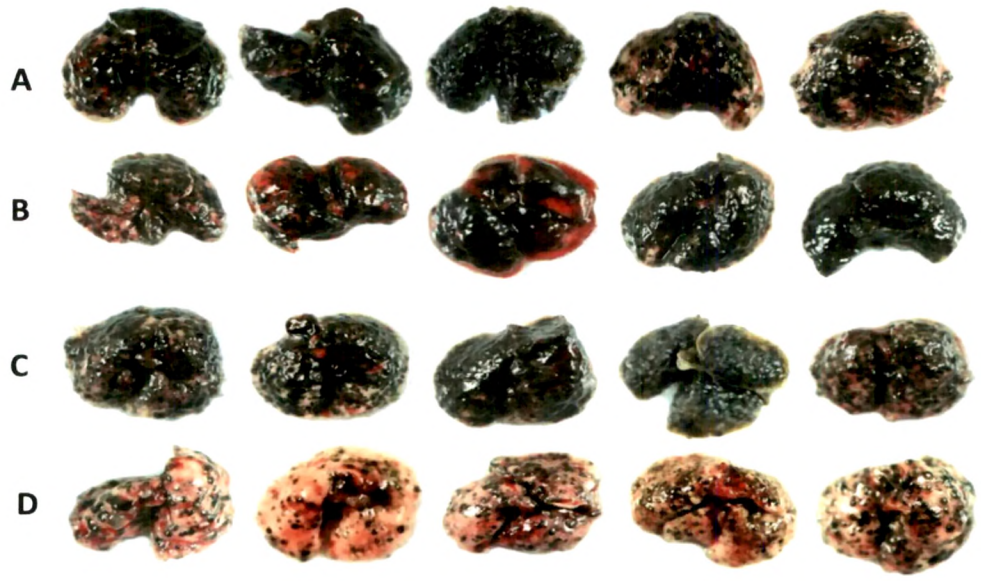


Figure 10.19. The appearance of the lungs from mice injected intravenously with highly metastatic B16-F10 melanoma cells ($1x10^5$) after intravenous administration of single dose of (A) untreated control (PBS); (B) Taxotere 40mg/kg; (C) Taxotere containing 10% 7-epidocetaxel; and (D) Taxotere containing 10% 10-oxo-7-epidocetaxel.

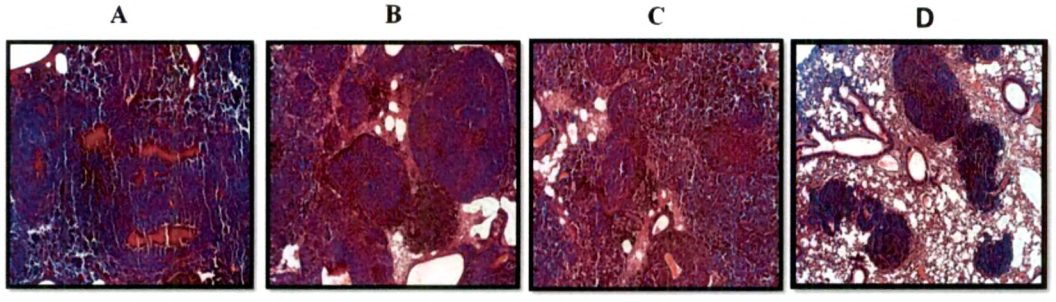


Figure 10.20. HE staining of the lungs from B16F10 melanoma metastasis bearing mice treated with single dose of (A) untreated control (PBS); (B) Taxotere 40mg/kg; (C) Taxotere containing 10% 7-epidocetaxel and (D): Taxotere containing 10% 10-oxo-7-epidocetaxel. Images were captured at X5 magnification.

10.11.8. Therapeutic study of 10-oxo-7-epidocetaxel

During single dose acute toxicity study, of Taxotere alone and Taxotere containing 10% docetaxel impurities, we observed surprisingly the decreased % average body weight loss and decreased number of surface metastatic nodules in mice treated with Taxotere containing 10% 10-oxo-7-epidocetaxel (at just 10%, 80μg/mouse) as compared to marketed Taxotere treated group (800μg/mouse) and Taxotere with 10% 7-epimer treated group. Therefore, we further evaluated the therapeutic effectiveness of the docetaxel impurity, 10-oxo-7-epidocetaxel, alone in mice bearing B16F10 pulmonary metastasis. We tested the therapeutic effectiveness of 10-oxo-7-epidocetaxel at single dose of 20mg/kg (400μg/mouse). The dose selected is half of the Taxotere tested dose in acute toxicity study. The dose was injected *via* tail vein at 9th day of B16F10 melanoma cell inoculation (1×10^5 cells/mouse). The cell concentration injected, day of drug administration (9th day) and day of animal scarification (20th day) are kept same as acute toxicity study to compare the results each other wherever it is possible.

About 8% mean body weight loss was observed with control group at the end of the experiment (on 20th day). The 10-oxo-7-epidocetaxel treated group shows no decrease in % body weight, instead the % mean group weight keep increased throughout the experiment and observed about 4% increased mean group weight at the end of the experiment (Table 10.6 and Figure 10.21). Therefore we can say that the weight loss of Taxotere with 10% 10-oxo-7-epidocetaxel treated group in acute toxicity study is because of toxicity of 90% (720μg/mouse) Taxotere injected and not because of 10% 10-oxo-7-epidocetaxel (80μg/mouse). Therefore we can conclude that the injected 10-oxo-7-epidocetaxel caused no toxicity (reduction in body weight) at the tested dose of 20mg/kg body weight.

No animals were found dead during the experiment. On 20th day the animals were sacrificed and lungs were excised and photographed (Figure 10.22). The metastatic nodules on lungs surface of the control group and 10-oxo-7-epidocetaxel treated group were counted to determine the therapeutic efficacy of 10-oxo-7-epidocetaxel in suppressing pulmonary B16F10 melanoma growth/metastasis. The numbers of surface metastatic nodules were found significantly less with 10-oxo-7-epidocetaxel treated group (107±49) (***$p<0.0001$) as compared to control group (348±56) (Figure 10.22 and 10.23). These results confirm the results obtained during acute toxicity study with Taxotere containing 10% 10-oxo-7-epidocetaxel.

The lungs of Taxotere treated group were found completely black and metastatic colonies were remain uncountable during the acute toxicity study at 40mg/kg dose. Therefore no therapeutic effect (no decreased metastatic nodules formation) was observed with Taxotere even at higher dose (40mg/kg) as compared to 10-oxo-7-epidocetaxel which showed significant decrease in metastatic nodules (suppressed significantly the pulmonary tumor growth/metastasis) at just half of the Taxotere dose (20mg/kg). Therefore the 10-oxo-7-epidocetaxel showed very significant and promising results (significantly decreased toxicity with increased therapeutic effect) than the

marketed Taxotere and further studies (including clinical studies) are needed to replace docetaxel with 10-oxo-7-epidocetaxel. The lung sections were fixed, stained with hematoxylin-eosin and observed under phase contrast microscope (Axio Imager. Z1 upright microscope, Germany). The images of control group showed large and fused nodules as compared to 10-oxo-7-epidocetaxel treated group which showed small and distinct nodules (Figure 10.24).

Table 10.6. The % group weights following administration of 20mg/kg single dose of 10-oxo-7-epidocetaxel to mice bearing B16F10 pulmonary metastasis

Day	Control (PBS)	10-oxo-7-epidocetaxel
0*	100±5.06	100±5.38
1	100.81±5.63	101.34±7.13
3	103.526.17	103.49±7.78
5	101.73±4.49	101.99±6.03
7	99.24±6.72	102.14±5.53
9	91.97±4.02	103.79±5.58

0*: Day when the drugs are administered,
Values represented are Mean ±SD

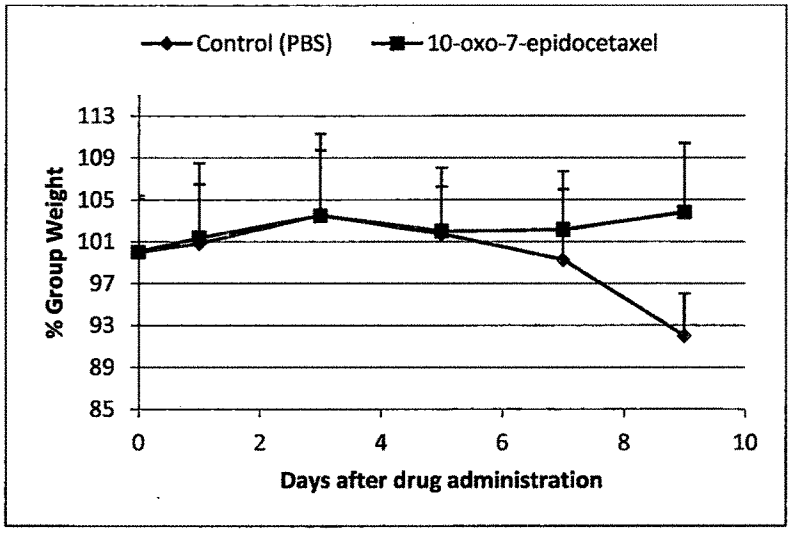


Figure 10.21. The % group weights following administration of 20mg/kg single dose of 10-oxo-7-epidocetaxel to mice bearing B16F10 pulmonary metastasis. The control group showed significant weight loss at the end of the experiment (20th day) (*$p<0.05$, $p=0.041$) as compared to 10-oxo-7-epidocetaxel treated group.

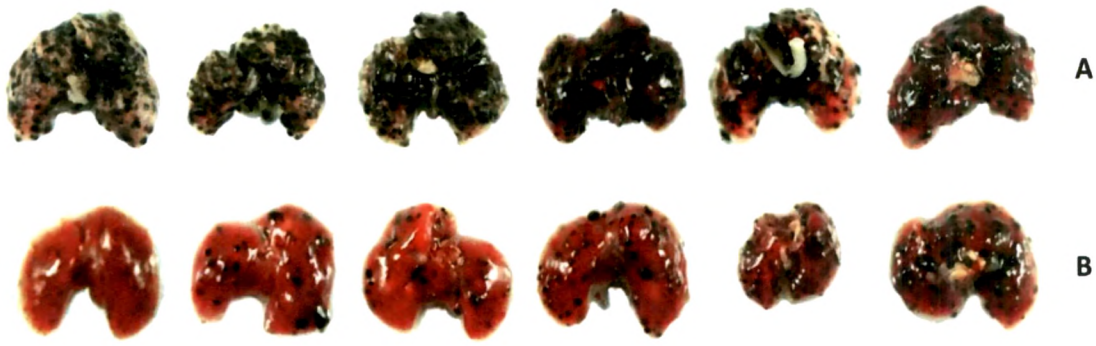


Figure 10.22. The appearance of the lungs from mice injected intravenously with highly metastatic B16F10 melanoma cells ($1x10^5$) after intravenous administration of single dose of (A) PBS (control); and (B) 10-oxo-7-epidocetaxel.

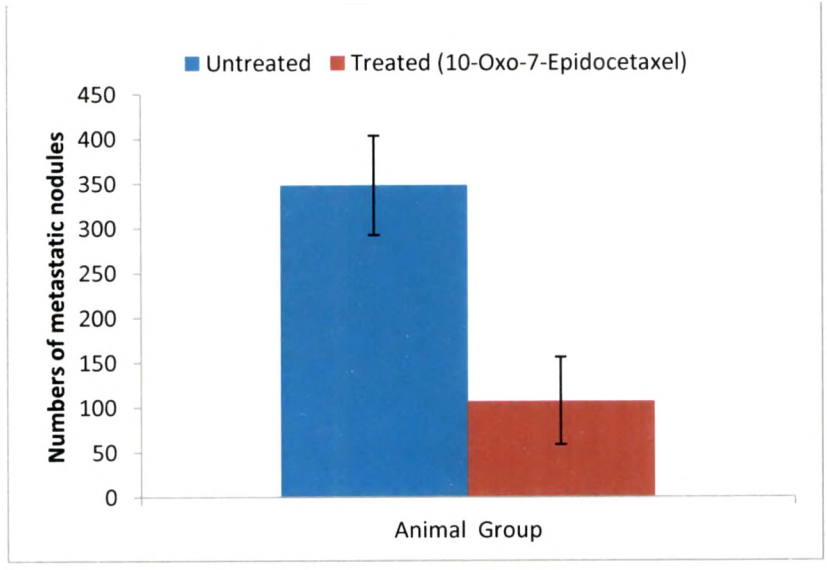


Figure 10.23. The suppression of pulmonary tumor growth/metastatic nodules in mice treated with 10-oxo-7-epidocetaxel at 20mg/kg single dose administered intravenously at 9th day of B16F10 melanoma cell inoculation ($1x10^5$/mouse) intravenously. The surface metastatic nodules were found significantly less with 10-oxo-7-epidocetaxel (***$p<0.0001$) treated group as compared to control group.

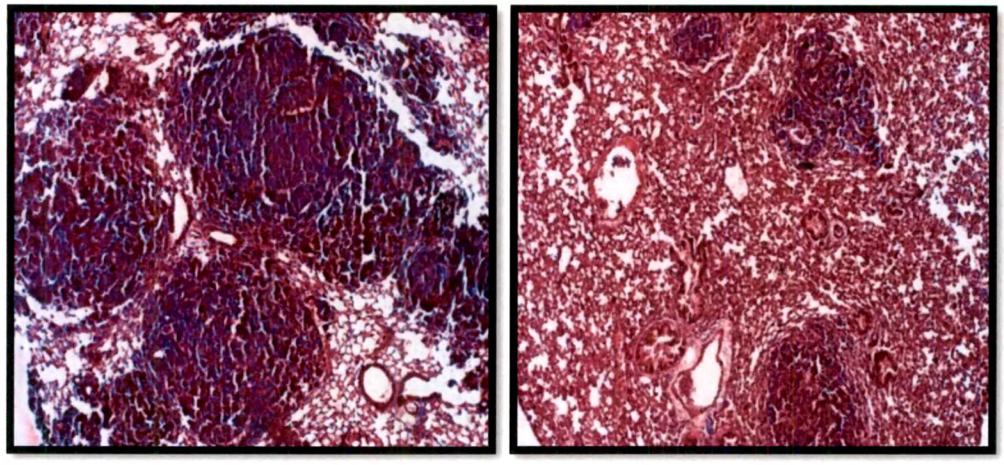


Figure 10.24. Images of mice lung sections after H-E staining (Left panel is untreated and Right panel is 10-oxo-7-epidocetaxel treated)

References

Bornique B, Lamarie A. Docetaxel (Taxotere) is not metabolised by recombinant human CYP1B1 *in vitro*, but acts as an effector of this isozyme. Drug Metab. Dispos. 2002; 30:1149-1152.

Chen L, Lu Y, Wua JM, Xua B, Zhang LJ, Gao M, Zheng SZ, Wang AY, Zhang CB, Zhang WW, Lei N. Ligustrazine inhibits B16F10 melanoma metastasis and suppresses angiogenesis induced by Vascular Endothelial Growth Factor. Biochem. Biophys. Research Commun. 2009; 386:374-379.

Chen Y, Zhu X, Zhang X, Liu B, Huang L. Nanoparticles Modified With Tumor-targeting scFv deliver siRNA and miRNA for cancer therapy. Mol. Ther. 2010; 18(9):1650-6.

Czejka M, Greil R, Ulsperger E, Schnait H, Kienesberger K, Brumnik T, Farkouh A, Schierholz J. Evidence For the Conversion of Docetaxel Into 7-Epidocetaxel in Patients Receiving Conventional Chemotherapy With Taxotere. Int. J. Clin. Pharmacol. Ther. 2010; 48:483-484.

Dora CL, Silva MA, Trentin AG, de Faria TJ, Fernandes D, da Costa R, Stimamiglio M, Senna EL. Evaluation of antimetastatic activity and systemic toxicity of camptothecin-loaded microspheres in mice injected with B16-F10 melanoma cells. J. Pharm. Pharmaceut. Sci. 2006; 9(1):22-31.

Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer 2002; 2(3):161-74.

Gaillard C, Monsarrat B, Vuilhorgne M, Royer I, Monegier B. Sabe S, Guenard D, Gires P, Archimbaud Y, Wright M, Sanderink GJ. Docetaxel (Taxotere) metabolism in the rat *in vivo* and *in vitro*. Proc. Amer. Assoc. Cancer Res. 1994, 35, 428 (abstract).

Gautam A, Waldrep JC, Densmore CL, Koshkina N, Melton S, Roberts L, Gilbert B, Knight V. Growth inhibition of established B16-F10 lung metastases by sequential aerosol delivery of p53 gene and 9-nitrocamptothecin. Gene Ther. 2002; 9:353-357.

Gueritte VF, Guenard D, Lavelle F, LeGoff MT, Mangatal L, Potier P. Relationship between the structure of taxol analogues and their antimitotic activity. J. Med. Chem. 1993; 34:992-998.

Guo CB, Wang S, Deng C, Zhang DL, Wang FL, Jin XQ. Relationship between matrix metalloproteinase 2 and lung cancer progression. Mol Diagn Ther. 2007;11(3):183-92.

Hwang PH, Yi HK, Kim DS, Nam SY, Kim JS, Lee DY. Suppression of tumorigenicity and metastasis in B16F10 cells by PTEN/MMAC1/TEP1 gene. Cancer Lett. 2001; 172:83-91.

Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I. Induction of Autophagic Cell Death in Malignant Glioma Cells by Arsenic Trioxide. Cancer res. 2003; 63:2103-2108.

Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010; 2;141(1):52-67.

Machado A, Fora JD, Maranduba A, Guimaraes E, Machado L, Marcio Santiago JR, Silva M. Pharmaceutical compositions containing docetaxel and a degradation inhibitor

and a process for obtaining the same. US Patent, US 20090221688 A1, September 3, 2009.

Mahoney BP, Raghunand N, Baggett B, Gillies RJ. Tumor Acidity, Ion Trapping and Chemotherapeutics I. Acid pH Affects the Distribution of Chemotherapeutic Agents *In vitro*. Biochem. Pharmacol. 2003; 66:1207-1218.

Monsarrat B, Royer I, Armand JP, Alvinerie P, DeForni M, Ho LK, Cresteil T, Wright M. 14th Europ. Workshop Drug Metabolism 1994, Paris B75, 274(abstract).

Nikkola J, Vihinen P, Vuoristo MS, Kellokumpu-Lehtinen P, Kähäri VM, Pyrhönen S. High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. Clin. Cancer Res. 2005; 15;11(14):5158-66.

Polette M, Nawrocki-Raby B, Gilles C, Clavel C, Birembaut P. Tumour invasion and matrix metalloproteinases. Crit. Rev. Oncol. Hematol. 2004;49(3):179-86.

Pöschl E, Schlötzer-Schrehardt U, Brachvogel B, Saito K, Ninomiya Y, Mayer U. Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. Development 2004;131(7):1619-28.

Ringel I, Horwitz SB. Taxol is converted to 7-epitaxol, a biologically active isomer, in cell culture medium. J. Pharmacol. Exp. Therapeutic. 1987; 242:692.

Salma J, McDermott JC. Suppression of a MEF2-KLF6 Survival Pathway by PKA Signaling Promotes Apoptosis in Embryonic Hippocampal Neurons. J. Neurosci. 2012; 32(8):2790-2803.

Sandoz AG. Pharmaceutical composition of improved stability containing taxane derivatives. European Patent, EP 1 946 747 A1, 2008.

Tannock IF, Rotin D. Acid pH in Tumors and Its Potential for Therapeutic Exploitation. Cancer Res. 1989; 49:4373-4384.

Vasu Dev R, Babu JM, Vyasa K, Sai Ramb P, Ramachandra P, Sekhar NM, Mohan Reddy DN, Srinivasa Rao N. Isolation and characterization of impurities in docetaxel. J. Pharm. Biomed. Anal. 2006; 40: 614-622

Vuilhorgne M, Gaillard C, Sanderink GJ, Monsarrat B, Dubois, J, Wright M, Royer I. 207th Amer. Chem. Soc. Meeting 1994; San Diego (abstract).

Wang S, Liu Q, Zhang Y, Liu K, Yu P, Liu K, Luan J, Duan H, Lu Z, Wang F, Wu E, Yagasaki K, Zhang G. Suppression of growth, migration and invasion of highly-metastatic human breast cancer cells by berbamine and its molecular mechanisms of action. Mol. Cancer 2009; 8:81.

Wright M, Monsarrat B, Royer I, Rowinsky EK, Donehower RC, Cresteil T, Guenard D. Metabolism and Pharmacology of Taxoids. Pharm. Libr. 1995; 22:131-164.

Zamir LO, Nedea ME, Belair S, Sauriol F, Mamer O, Jacqumain E, Jean FI, Garneau FX. Taxanes isolated from Taxus canadensis. Tetrahedron Lett. 1992; 33:5173-5177.