

## RESEARCH ARTICLE

# Nuclear DNA Damage and Repair in Normal Ovarian Cells Caused by Epothilone B

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### Abstract

This study was designed to assess, whether a new chemotherapeutic microtubule inhibitor, Epothilone B (EpoB, Patupilone), can induce DNA damage in normal ovarian cells (MM14.Ov), and to evaluate if such damage could be repaired. The changes were compared with the effect of paclitaxel (PTX) commonly employed in the clinic. The alkaline comet assay technique and TUNEL assay were used. The kinetics of DNA damage formation and the level of apoptotic cells were determined after treatment with IC50 concentrations of EpoB and PTX. It was observed that PTX generated significantly higher apoptotic and genotoxic changes than EpoB. The peak was observed after 48 h of treatment when the DNA damage had a maximal level. The DNA damage induced by both tested drugs was almost completely repaired. As EpoB in normal cells causes less damage to DNA it might be a promising anticancer drug with potential for the treatment of ovarian tumors.

**Keywords:** Apoptosis - DNA damage - Epothilone B - Paclitaxel

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### Introduction

Drugs able to induce DNA damage, such as microtubule inhibitors, play a critical role in DNA replication or in the segregation of chromosomes during cell division (Attia et al., 2013; Ji et al., 2014). One of the compounds with these properties is PTX, which belongs to the taxane group and is utilised in the treatment of a variety tumor types (Sharifi et al., 2014; Wang et al., 2014; Zhang et al., 2015). PTX stabilizes the microtubule polymers and protects them from disassembling. The chromosomes are thus unable to achieve a metaphase spindle configuration. This blocks the progression of mitosis. Further, a prolonged activation of the mitotic checkpoint triggers either an apoptosis or a reversion to the G-phase of the cell cycle without cell division (Zasadil et al., 2014). A big problem in the successful treatment with the taxanes is the appearance of the primary and acquired resistance, which has driven the search for alternative agents, such as epothilones (EPOxide, THIazoLe, ketONES), which could replace them (Smaglo and Pishvaian, 2014; Navarrete et al., 2015).

Patupilone is a natural compound consisting of the common macrolide ring with a methylthiazole side chain. This and other natural epothilones are produced by the mixobacterium *Sorangium cellulosum* (Osswald et al., 2014; Gong et al., 2015). Epothilone B exerts higher potency than paclitaxel in cancer cells (6-25-fold) (Bollag et al., 1995; Rogalska et al., 2013b). Biological studies have shown that epothilones stabilize microtubules in the same way as paclitaxel, but with slightly higher efficiency.

Epothilones bind to the same site on  $\beta$ -tubulin as PTX. They share one polar place at C7-OH, whereas the thiazole side chain is associated with a different region of  $\beta$ -tubulin than the taxanes (Goodin, 2008). For these reasons, EpoB competitively inhibits the binding of PTX to microtubules (Sharma et al., 2013).

Epothilones are nonselective and exert cytotoxic activity against cancer and normal cells alike. Anticancer drugs can initiate genotoxic stress, which leads to DNA damage and apoptosis in nontumor cells, which in turn may lead to the development of secondary tumors from cells that were not originally neoplastic (Attia, 2013; Rogalska et al., 2013c). Because the influence of EpoB on DNA damage in nontumor cells has not been reported yet, it is important to investigate the genotoxic effect of EpoB on normal cells. Hence, the aim of the current study was to investigate DNA damage, its capability to repair and apoptosis after induction by EpoB in comparison to PTX in normal mouse ovarian cells. In addition, the possible mechanism underlying this modulation was assessed. The kinetics of the repair of the DNA damage was evaluated using the comet assay. Apoptosis was analyzed using the TUNEL assay.

### Materials and Methods

#### Chemicals

PTX was obtained from Sequoia Research Products (Pangbourne, United Kingdom). EpoB and trypsin-EDTA were purchased from Sigma (St. Louis, USA). The

medium (DMEM) and fetal bovine serum (FBS) were supplied by Cambrex (Basel, Switzerland). Apo-BrdU In Situ DNA Fragmentation Assay Kit was supplied by BioVision Inc. (Milpitas, California, USA). All other chemicals and solvents were of high analytical grade and were obtained from Sigma (St. Louis, USA).

#### Cells cultures and treatment

MM14.Ov (mouse normal ovarian cell line) was obtained from the American Type Culture Collection (Rockville, MD, USA). MM14.Ov cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (10 U/mL), and streptomycin (50 µg/mL) under standard conditions: 37°C, 100% humidity, the atmosphere being 5% CO<sub>2</sub> and 95% air. The cytotoxic activity of EpoB and PTX was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test described previously (Rogalska et al., 2013c). The IC<sub>50</sub> values were 70.46 nM and 1 µM for EpoB and PTX, respectively. To compare the genotoxic and apoptotic effects of both drugs, the IC<sub>50</sub> of the drugs in all tests was used. Drugs were added and the cells were incubated in a CO<sub>2</sub> incubator for different periods of the time (2–48 h), depending on the testing method.

#### Comet assay

The comet assay was performed under alkaline conditions according to the procedure of Singh and colleagues with minor modifications (Singh et al., 1988). The control cells and cells treated with EpoB or PTX were collected at 2, 4, 24 and 48 h after culture initiation. For the repair assay, the cells were allowed to recover from the induced damage by being washed in PBS and incubated at 37°C with fresh media for 30, 60 and 120 min before being harvested. Fifty randomly selected cells from each slide were measured using the image analysis (Nikon, Japan) attached to a COHU 4910 video camera (USA), which was equipped with a UV-1 filter block consisting of an excitation filter (359 nm) and a barrier filter (461 nm) connected to the image analysis system Lucia-Comet v. 4.51 (Czech Republic). The % of DNA in comet tail was estimated for the presentation of the results.

#### TUNEL assay

Drug-treated and the control cells were processed according to the Apo-BrdU In Situ DNA Fragmentation Assay Kit protocol supplied by the manufacturer (BioVision). The cell fluorescence was measured with flow-cytometry (Becton Dickinson equipped with an UV-argon laser). The green fluorescence of FITC at 520 nm and the red fluorescence of propidium iodide at 623 nm were detected. A number of TUNEL positive cells was expressed as a percentage of the total number of cells in the sample.

#### Statistical analysis

Analysis of variance (ANOVA) with the Tukey post hoc test was used for multiple comparisons. All statistical tests were calculated using the Statistica software (StatSoft, Tulsa, OK, USA), and the significance level

was set at p-values of <0.05.

## Results

#### Measurement of DNA damage

The quantitative results obtained clearly demonstrate that EpoB, similarly to PTX, is able to induce the DNA damage in MM14.Ov cells (Figure 1). The observed increase in DNA damage after 2–48 h of incubation with both drugs was time-dependent and statistically significant. The highest percent of the DNA in the tail was observed after 24h of PTX treatment (55.22%). EpoB after 24h of incubation mediated smaller DNA damage at the level of 24.31%.

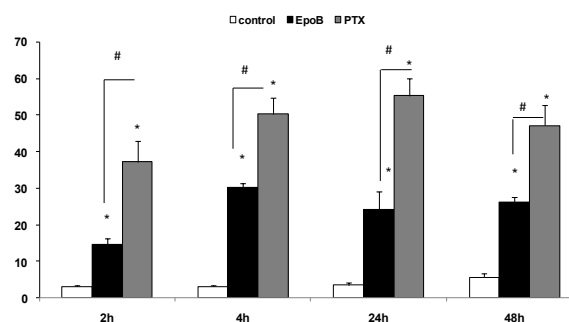
The level of the DNA damage was higher for PTX than that obtained for EpoB in all investigated times of incubation. The differences between the drugs, in a percent of DNA in tail, were statistically significant. After 2 and 4h of treatment the differences were about 20%, maximum was at 24h of incubation with drugs (31%), and after 48 differences reached level of 21%.

#### DNA repair

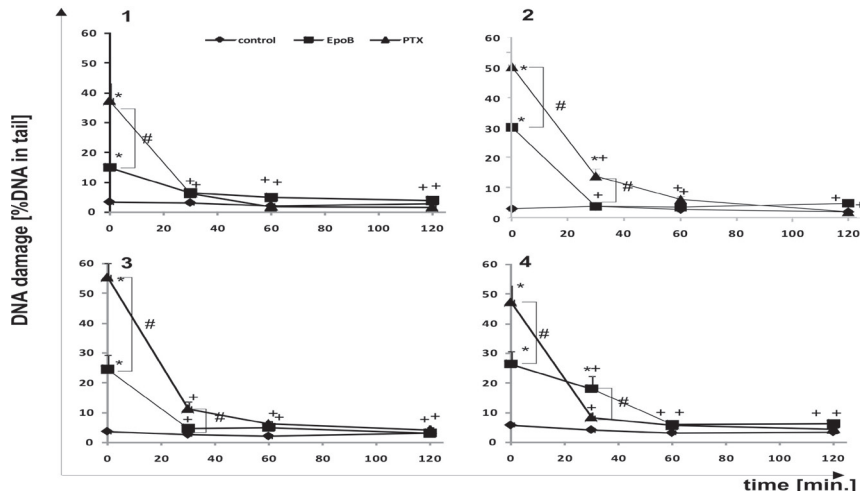
Figure 2 displays the kinetics of the DNA damage repair. There were not time-dependent significant differences in the mean efficacy of the DNA repair between EpoB or PTX treated cells, even in the case of cells incubated in the drug free medium up to 120 min. When the drug was removed from the medium the percentage of the DNA in tail drastically decreased to the level below 10%.

#### Analysis of apoptosis

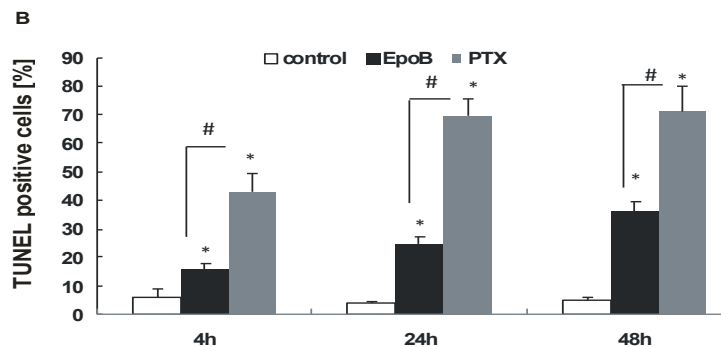
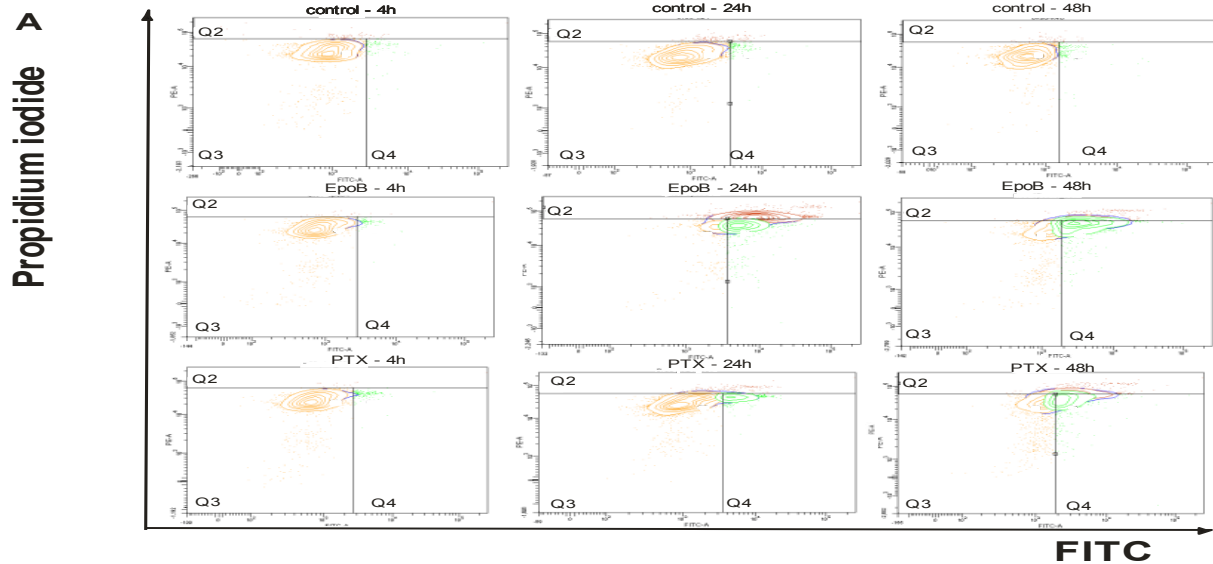
As shown in Figure 3, EpoB increased the level of TUNEL-positive cells in a time-dependent manner. The maximum increase in the percentage of apoptotic cells with DNA strand-breaks was detected after 48h of treatment with EpoB (35.39%). PTX induced higher percentage of apoptosis at all incubation times. The changes in the level of TUNEL-positive cells induced by EpoB were



**Figure 1. Endogenous DNA Damage.** Mean basal endogenous DNA damage measured as the mean comet tail DNA of control cells (without visual disturbances) which were treated with IC<sub>50</sub> concentration of EpoB and PTX. The number of analyzed cells in each treatment was 100 and the analysis was repeated four times. Error bars denote SEM. (\*) Differences statistically significant in comparison to control cells, p<0.05; (#) Statistically significant differences observed between the probes incubated with EpoB in comparison to the effect after treatment with PTX



**Figure 2. DNA Damage Repair.** Representative kinetics of DNA damage repair in MM14.Ov cells. DNA damage was induced by incubation with IC50 concentration of EpoB and PTX for (1) 2h, (2) 4h, (3) 24h and (4) 48h. Repair incubation was in a fresh medium after washing out drugs for 30, 60 and 120 min. Error bars denote SEM. (\*) Differences statistically significant in comparison to control cells,  $p < 0.05$ ; (#) Statistically significant differences observed between the probes incubated with EpoB in comparison to the effect after treatment with PTX,  $p < 0.05$ . (+) Statistically significant differences observed between the probes incubated with drugs in comparison to the effect after washing out drugs at 30, 60 and 120 min,  $p < 0.05$



**Figure 3. Apoptosis Detection.** Induction of apoptosis in MM14.Ov cells after treatment with IC50 EpoB or PTX concentration evaluated by TUNEL assay. A) Typical cytometric histogram obtained after 4h, 24 h and 48 h of incubation with the drugs. Q2-TUNEL positive cells, PI positive; Q3-TUNEL negative, PI negative cells; Q4-TUNEL positive, PI negative cells. B) Quantitative results of the effect of drugs on the level of TUNEL positive cells. Results represent mean  $\pm$  SD of four independent experiments. (\*) Values statistically significant in comparison to control cells. (#) Statistically significant differences observed between the probes incubated with EpoB in comparison to the effect after treatment with PTX,  $p < 0.05$

significantly lower than that of PTX and the mean ratios of values for 2h, 24h and 48h of incubation were 2.7, 2.8,

and 2 respectively (Figure 3). For all incubation times the differences between drugs were statistically significant.

## Discussion

Microtubules are promising targets for chemotherapeutic drugs aimed at disturbing mitosis and inducing cell death in frequently dividing tumor cells (Wu et al., 2014). EpoB, due to its high cytotoxicity in ovarian cancer cells, seems to be a very promising alternative to the current strategy of ovarian cancer treatment, currently in which PTX is the main component (Unal et al., 2014; Vahdat et al., 2013). Although, the efficacy and toxicity of the epothilones in the cancer cells were confirmed, their future role in this therapy remains to be appropriately defined to include their effects on normal cells (Aogi et al., 2013; Gauler et al., 2013; Roque et al., 2013). The evaluation of not only the cytotoxic but also the genotoxic impact of EpoB on normal ovarian cells grows in importance.

It is well known that DNA damage plays fundamental role in the mechanism of taxanes action. The degradation of DNA can be caused by single and double stranded DNA breaks that arise from the generation of free radicals (Raj et al., 2014). The free radicals can lead to the DNA damage and instability of genome. It has been reported that PTX is a somatic cell genotoxic agent and is able to induce chromosomal damage (Zhang et al., 2012; Attia et al., 2013).

Our previous results clearly demonstrate that Epo B and PTX were able to generate ROS in MM14.Ov cells. We showed that PTX caused significant increases in DNA damage formation in MM14.Ov cells. PTX caused increases in all comet parameters with the highest values at 24h of PTX exposure. One of the most important aspects of presented results was a significant lower level of endogenous DNA damage in MM14.Ov cells treated with EpoB in comparison to PTX. This result is of great importance especially in the context of our earlier studies, which show that EpoB was more cytotoxic in cancer cells than PTX (Rogalska et al., 2013c). We also evaluated the total levels of the protein p53. P53 is an important protein, as it plays a critical role in the apoptosis of irreversibly damaged cells (Stengel et al., 2014). Being a tumor suppressor, it is associated with the molecular mechanism of chemotherapy resistance. P53 localizes to microtubules (MT) and, in response to DNA damage, is transported to the nucleus via the MT minus-end-directed motor protein dynein. Our result showed that EpoB increases the level of p53 earlier than PTX, and the amount of p53 returns to the control value at the 24 h time point. The level of p53 after PTX treatment continued to be high (Rogalska et al., 2013a). P53 is stabilized and activated after DNA damage. However, the cascade of events from DNA damage to the p53 stabilization and activation is still controversial.

The cleavage of PARP (Rogalska et al., 2013a) further confirms that in the tested drugs a DNA damage is observed. Poly (ADP- ribosylation) of different nuclear acceptors by PARP-1 is an early event when a single strand DNA lesion is produced. The role of PARP-1 in the p53-mediated response to the DNA damage is dependent on the type of DNA lesion and on the upstream pathway leading to the p53 activation (Ireno et al., 2014).

EpoB can accumulate in the cell nucleus, specifically in the fraction of nuclear proteins. This indicates an

inhibition of some proteins, such like the nuclear tubulin isotype, therefore resulting in a possible DNA-damaging effect of epothilone B (Lichtner et al., 2001; Baumgart et al., 2015). In addition to the genetic damage, the DNA repair and its susceptibility to apoptosis are factors which play an important role in the development of cancer. The kinetics of repair of DNA damage in ovarian cells treated with PTX and EpoB were evaluated. To our knowledge there are no studies in which the comet assay has been used to assess DNA repair after damage induced by EpoB in normal ovarian cells. DNA repair took place at the similar level after both drugs treatment in tested cells. Further studies will be necessary to elucidate the mechanisms of DNA repair involved in the EpoB-induced DNA damage.

DNA fragmentation occurs as one of the final stages of cell death and is considered a hallmark of apoptosis and one of the defining biochemical events of the pathway. To examine whether EpoB and PTX induce apoptosis by generating DNA strand breaks, ovarian cells were stained using TUNEL assay. Both drugs induce TUNEL-positive cells in time-dependent manner. It is well known that PTX produces apoptosis in tumor and nontumor cells (Rogalska et al., 2013a; Lalli et al., 2015). In agreement with these cited reports, the data presented here indicate that PTX lead to a higher level of apoptotic cells than EpoB at all measured time points, and it correlates well with the level of the fragmented DNA detected by comet assay. Similar conclusion was also obtained by studies investigating the mechanism of sagopilone (SAG) action. The SAG-induced increase in DSBs is not direct effect of SAG, but rather the consequence of the increased apoptosis (Winsel et al., 2011; Goldar et al., 2015). However, the role of DNA damage in response to EpoB and its potential role in the activity of EpoB in normal ovarian cells will need further investigation.

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