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Preclinical report

In vitro sensitivity of normal human mesothelial and malignant mesothelioma cell lines to four new chemotherapeutic agents

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In this study, we used four human mesothelioma cell lines (M14K, M24K, M25K and M38K), one transformed human mesothelial cell line (MeT-5A) and one primary mesothelial culture (UPL) to test for *in vitro* sensitivity to docetaxel, paclitaxel, SN-38 [an active metabolite of irinotecan (CPT-11)] and gemcitabine, as single agents. Subconfluent cell cultures were treated with 2×10^{-9}, 5×10^{-9}, 10^{-8}, 2×10^{-8} and 5×10^{-8} M concentrations of each drug for 48 h. The sensitivity was measured in terms of cell viability using the Trypan blue exclusion method. All four drugs were potent inhibitors of mesothelioma cell growth, but cell lines from different patients diverged in their sensitivity to the individual agents. In most cases docetaxel, paclitaxel and SN-38 were more potent killers of mesothelioma cells than gemcitabine. The induction of DNA damage was investigated using the Comet assay; cells from two cell lines (M14K and M25K) were treated with subtoxic 10^{-8} M concentrations of each drug for 4, 24 and 48 h. Each of the agents caused a slight increase in DNA single-strand breaks at a concentration of 10^{-8} M. [© 2000 Lippincott Williams & Wilkins.]

Key words: Comet assay, docetaxel, gemcitabine, *in vitro* sensitivity, mesothelioma, paclitaxel, SN-38.

Introduction

Malignant mesothelioma originates from the mesothelial cells surrounding the pleural and peritoneal cavities. About 80% of patients have a history of occupational exposure to asbestos. Prognosis is rather poor; median survival being 8–20 months depending on prognostic factors and treatment.[1] There is no standard treatment for malignant pleural mesothelioma. Surgery, chemotherapy and radiotherapy have been used, either alone or in combination, with disappointing results. There is a particular need to study new chemotherapeutic agents, both *in vitro* and in clinical trials, to improve treatment results.[2,3] It is, however, difficult to screen new drugs and their different combinations in clinical trials, in a rare disease such as malignant mesothelioma. *In vitro* testing has been suggested as a useful tool for assessing the sensitivity of mesothelioma cells to new drugs before clinical trials.

In our previous *in vitro* studies we showed that methotrexate had a high level of activity against mesothelioma cell lines[4] and this activity was confirmed in the clinical setting.[5,6] We, therefore, planned the present study to test the sensitivity of four mesothelioma cell lines to four new chemotherapeutic agents, docetaxel (Taxotere®) and paclitaxel (Taxol®) which are mitotic spindle poisons, and SN-38 [an active metabolite of irinotecan (CPT-11)] and gemcitabine (Gemzar®), which are both involved in the inhibition of DNA synthesis. A transformed human mesothelial cell line (MeT-5A) and a primary mesothelial cell culture (UPL) were included in the study for reference. We were interested in finding an optimal concentration that would affect the mesothelioma cells and furthermore in investigating whether these agents caused DNA single-strand breaks (SSBs). We also wanted to study the DNA SSBs and their possible repair at different points in time. The viability of the cells was assessed using the Trypan blue exclusion

SN-38 and docetaxel were supplied by Rhône-Poulenc Rorer, Antony, France.

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method and the DNA damage caused by the four agents was investigated by single-cell gel electrophoresis (the Comet assay).

Materials and methods

Cell cultures

Previously established mesothelioma cell lines (M14K, M24K, M25K and M38K) were used in the study.[7] Cell line M14K was established from an epithelial subtype tumor, whereas the other three cell lines were all grown from mixed subtype tumors. The cells were grown in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Eggenstein, Germany), 0.03% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Paisley, UK). The cell lines had similar growth rates, with an average doubling time of 30 h. Primary mesothelial cell cultures were established from non-malignant pleural effusions from patients treated at the Helsinki University Central Hospital (heart failure or inflammatory disease). The fluid was centrifuged (1500 r.p.m., 5 min) and the pelleted cells were washed with growth medium and then transferred to tissue culture flasks. These cells were grown in RPMI 1640 medium containing 20% FBS, other supplements being the same as for the mesothelioma cells. Transformed human mesothelial (MeT-5A) cells (provided by Dr C Harris, National Cancer Institute, Laboratory of Human Carcinogenesis, Bethesda, MD) were cultured under the same conditions as the mesothelioma cell lines. These non-tumourigenic MeT-5A cells are SV-40 virus-transformed, immortalized, human mesothelial cells with typical mesothelial morphology.[8] The average doubling time for MeT-5A cells is about 30 h. All the cell lines were incubated at 37°C in a humidified atmosphere with 5% CO_2.[7,9]

Exposure to chemotherapeutic agents

Exponentially growing monolayer cell cultures were detached using trypsin and plated into six-well plates (Nunc, Roskilde, Denmark) in 3 ml growth medium, for the Trypan blue and Comet assays. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 48 h in order to re-establish exponentially growing cultures. The semi-confluent cell cultures were then treated with docetaxel (Rhône-Poulenc Rorer, Antony, France), paclitaxel (Bristol-Myers Squibb, Syracuse, NY), SN-38 (Rhône-Poulenc Rorer) or gemcitabine (Lilly, Mount-Saint-Guibert, Belgium), at concentrations of 2×10^{-9}, 5×10^{-9}, 10^{-8}, 2×10^{-8} and 5×10^{-8} M for 48 h for the cell viability studies. A subtoxic concentration of 10^{-8} M, as established in the cell viability tests, was used in the Comet assay for 4, 24 and 48 h exposures. Treatment media were the same as the growth media except for the 4 h exposures during which no supplements were added to the medium.

Cytotoxicity

After the 48 h exposure, cells were collected by trypsinization and the number of living cells remaining was then assessed using the Trypan blue exclusion method. Two to three independent sets of experiments with duplicate cultures were completed for each drug. Viability was expressed as the percentage of living cells in the test cultures compared to the unexposured control cultures (=100%) set up in each experiment.

The Comet assay

Induction of DNA SSBs by the Comet assay was performed on two mesothelioma cell lines, M14K and M25K, and on the transformed human mesothelial cell line, MeT-5A, at three different time points of exposure, after 4, 24 and 48 h. The Comet assay was performed under alkaline conditions (pH 13) according to the method of Singh *et al.*[10] with slight modifications. After exposure the cells were detached using trypsin, centrifuged and re-suspended in pre-heated (37°C) 0.5% low melting point agarose (LMPA; BioRad, Hercules, CA). The cell suspension was put on to dry microscope slides (Assistant, Sondheim/Rhön, Germany), precoated with 1% normal melting agarose (International Biotechnologies, New Haven, CT) and allowed to harden for 10 min at 4°C. The slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% Triton X-100) for at least 1 h at 4°C. The slides were then placed in an electrophoresis tank and DNA was allowed to unwind for 20 min in the freshly made alkaline electrophoresis buffer (1 mM EDTA and 300 mM NaOH). Electrophoresis was conducted at room temperature for 15 min at 24 V and 300 mA. The slides were then neutralized 3 times with Tris buffer (0.4 M, pH 7.5) and stained with ethidium bromide. All the steps were performed under yellow light in order to prevent additional DNA damage. The slides were examined using a fluorescent microscope attached to a CCD camera connected to a personal computer-based image analysis system (Komet 4.0; Kinetic Imaging, Liverpool, UK). A total of 800 cells from two separate experiments (with duplicate

cultures) was scored (200 cells from each culture). Single cells were analyzed under the fluorescence microscope. DNA SSBs were measured as the mean of the tail moments. Tail moments were calculated as the tail length multiplied by the percentage of DNA in the tail. Exposure to 100 μM H_2O_2 for 30 min was used as a positive control through the Comet assay study.

Statistical analysis

Data are expressed as mean $\pm$ SE from two to three separate experiments. Two groups were compared using a two-tailed Student's *t*-test. $p < 0.05$ was considered to be significant.

Results

Cytotoxicity

Docetaxel. All four mesothelioma cell lines showed a dose-related response to docetaxel after the 48 h treatment. The cell line M25K was the most sensitive to this agent, at a concentration of 10^{-8} M (20% viability). The most resistant cell line, M24K, had a cell viability of 56% under the same conditions (Table 1). M25K cell viability was less than 50% at all doses of docetaxel, whereas for M24K, cell viability stayed above 50% for all doses. Exposure to concentrations of docetaxel of 10^{-8} M or higher resulted in cell viability levels of less than 50% in the M14K and M38K mesothelioma cell lines. The non-malignant control cells were sensitive to all doses of docetaxel. MeT-5A cells were slightly more sensitive than UPL cells (Figure 1a).

Paclitaxel. Paclitaxel had the most potent effect on the M38K mesothelioma cell line, in which a concentration of 10^{-8} M reduced cell viability to 34%. M14K cells were the most resistant to paclitaxel at this concentration (57% viability). Paclitaxel at concentrations of 10^{-8} M or higher reduced cell viability to less than 50% in the M25K and M38K cell lines. In the M14K cell line only a concentration of 5×10^{-8} M reduced cell viability to less than 50%. Cell viability in the M24K cell line was slightly above 50% at all the doses tested (Table 1). Non-malignant cell lines were sensitive to paclitaxel at a concentration of 10^{-8} M. The MeT-5A cell line was again observed to be a little more sensitive than UPL cells (Figure 1b).

SN-38. All the mesothelioma cell lines responded to SN-38 at the given doses. SN-38 was most toxic in the M14K and M25K cell lines; the percentage of viable cells after exposure to a concentration of 10^{-8} M was 13 and 14%, respectively. The M24K cell line was the most resistant to this agent, 93% of cells remaining viable at a concentration of 10^{-8} M. Only exposure to a concentration of 5×10^{-8} M reduced cell viability to less than 50% in the M38K cell line (Figure 1c). MeT-5A and UPL cells showed very similar sensitivities to SN-38, especially at a concentration of 10^{-8} M (Table 1).

Gemcitabine. At a concentration of 10^{-8} M gemcitabine had little effect on any of the cell lines, including the non-malignant cells. The M14K and M25K cell lines were especially resistant to this agent. M24K and M38K were more sensitive to gemcitabine (Table 1). Only the highest concentration (2×10^{-8} M) reduced cell viability to less than 50% in the MeT-5A cell line (Figure 1d).

Further analysis. We placed all agents into the order of potency at a concentration of 10^{-8} M (Table 2). Gemcitabine was the least active agent at the given doses, whereas the taxanes were the most active in non-malignant cell lines. Docetaxel was superior to paclitaxel in the M14K and M25K cell lines, while paclitaxel was more active in the M24K and M38K cell lines.

The Comet assay

A small but significant increase in the mean tail moment was detected after a 4 h exposure to 10^{-8} M of docetaxel in both mesothelioma cell lines (M14K and M25K) and in MeT-5A cells. SN-38 produced a significant increase in mean tail moment in M25K cells after a 4 h exposure. The same exposure to gemcitabine caused a significant increase in mean tail moment in M14K cells. Paclitaxel caused a significant increase in the mean tail moment in MeT-5A cells but not in either mesothelioma cell lines at this time point (Figure 2a).

All four chemotherapeutic agents caused significant increases in mean tail moment in both mesothelioma cell lines when the exposure time was increased to 24 h. At this exposure time, the greatest increase in tail moment in M14K cells was caused by docetaxel and in M25K cells the most potent DNA-damaging agent was paclitaxel. All the other drugs induced significant DNA damage in MeT-5A cells except docetaxel (Figure 2b).

In M14K cells, no DNA damage was detected after 48 h exposures to any of the four chemotherapeutic agents. However, in M25K cells, the 48 h exposure to gemcitabine or paclitaxel caused significant increases

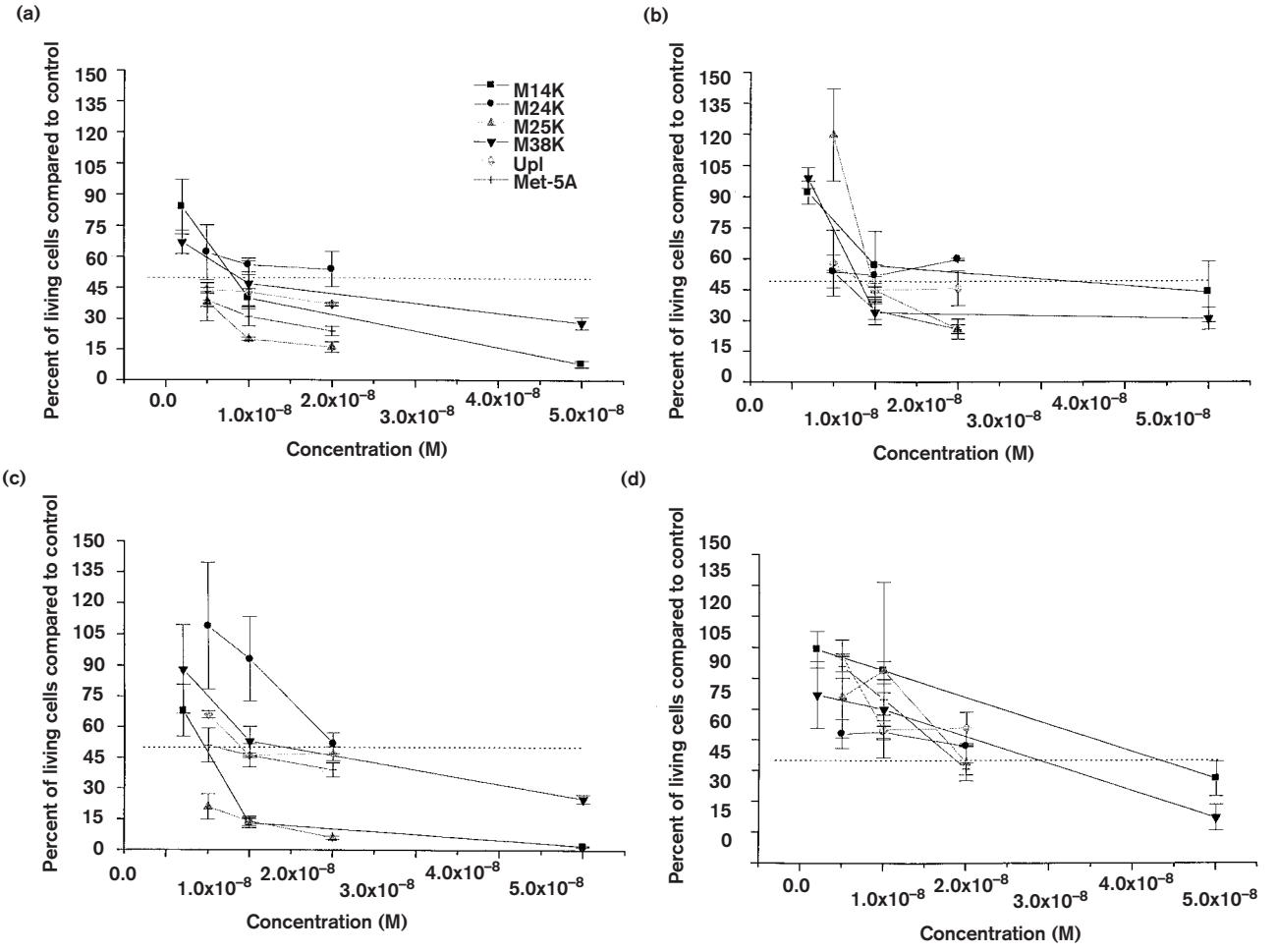

Figure 1. Percentage of living cells remaining after exposure to 2×10^{-9}, 5×10^{-9}, 10^{-8}, 2×10^{-8} and 5×10^{-8} M concentrations of docetaxel (a), paclitaxel (b), SN-38 (c) and gemcitabine (d) for 48 h, in four mesothelioma cell lines (M14K, M24K, M25K and M38K), in primary mesothelial cells (UPL) and and in a transformed mesothelial cell line (MeT-5A). Values are means $\pm$ SEM from two to three experiments. $^*p<0.05$.

Table 1. The percentage of living cells surviving, compared to control, after a 48 h exposure to four new chemotherapeutic agents (at 10^{-8} M) (SEs from three different experiments are presented in parentheses)

	M14K	M24K	M25K	M38K	UPL	MeT-5A
Docetaxel	40 (4.4)	56 (3.3)	20 (0.8)	47 (10.9)	43 (8.3)	31 (4.6)
Paclitaxel	57 (16.4)	52 (5.5)	45 (6.6)	34 (5.9)	45 (3.4)	35 (4.2)
SN-38	13 (2.3)	93 (20.3)	14 (2.2)	53 (7.1)	46 (5.6)	46 (1.0)
Gemcitabine	94 (4.4)	64 (3.1)	94 (42.6)	75 (8.3)	65 (4.7)	80 (7.6)

in mean tail moment. Gemcitabine was the only agent that caused a significant increase in tail moment in MeT-5A cells at this exposure (Figure 2c).

Discussion

The number of new and interesting chemotherapeutic agents has rapidly increased over the past few years, giving hope of more effective regimes for treating mesothelioma. In order to identify new chemotherapeutic agents which might be effective for clinical use, either singly or in combination, it is crucial to understand their mechanisms of action. Two of the agents we used, docetaxel and paclitaxel, are mitotic spindle poisons, and the other two, SN-38 and

Table 2. Activity pattern sequences for four agents at the concentration of 10^{-8} M using the Trypan blue exclusion method

Cell line	Sequence of activity pattern
M14K	S>D>P>G
M24K	P>D>G>S
M25K	S>D>P>G
M38K	P>D>S>G
MeT-5A	D>P>S>G
UPL	D>P>S>G

D, docetaxel; P, paclitaxel; S, SN-38; G, gemcitabine.

gemcitabine, are extensively involved in the inhibition of DNA synthesis.

Docetaxel and paclitaxel both induce uncontrolled tubulin polymerization, but there are some specific quantitative differences between them. Docetaxel has been shown to be more active as a tubulin assembly promoter and a microtubule stabilizer, and more potent inhibitor of microtubule depolymerization, than paclitaxel.[11,12] Docetaxel has been found to be twice as efficient as paclitaxel in decreasing the minimum concentration of tubulin required for microtubulin assembly.[13] An important finding for clinical use, with respect to schedules of administration, is that docetaxel and paclitaxel seem to be active during different phases of the cell cycle. Docetaxel is mainly active during the S phase, whereas paclitaxel is most often active late in the G_2/M phase. Docetaxel is also more cytotoxic than paclitaxel in the majority of human primary tumour specimens.[14] In the cell viability tests, docetaxel was more cytotoxic than paclitaxel in two of the four mesothelioma cell lines (M14K and M25K) but the other two cell lines (M24K and M38K) were more sensitive to paclitaxel. The results of the Comet assay suggest that docetaxel and paclitaxel are equally potent agents for inducing DNA damage in mesothelioma cell lines. This suggests that the Comet assay results were not comparable to the 48 h cell viability test. In the non-malignant cell lines, docetaxel and paclitaxel caused DNA damage during exposures of up to 24 h, but with a longer exposure time DNA damage was not apparent. This could be due to DNA repair or to the loss of the most heavily damaged cells.

CPT-11 interferes with DNA replication by inhibiting the DNA topoisomerase I enzyme and then provoking a single, followed by a double, strand break forcing the cell to enter apoptosis. The cytotoxic effect of CPT-11 appears to depend on exposure time.[15] SN-38 is a water-insoluble metabolite of CPT-11 and it is considered to be the active metabolite most responsible for the cytotoxic effects.[16] Our results confirmed

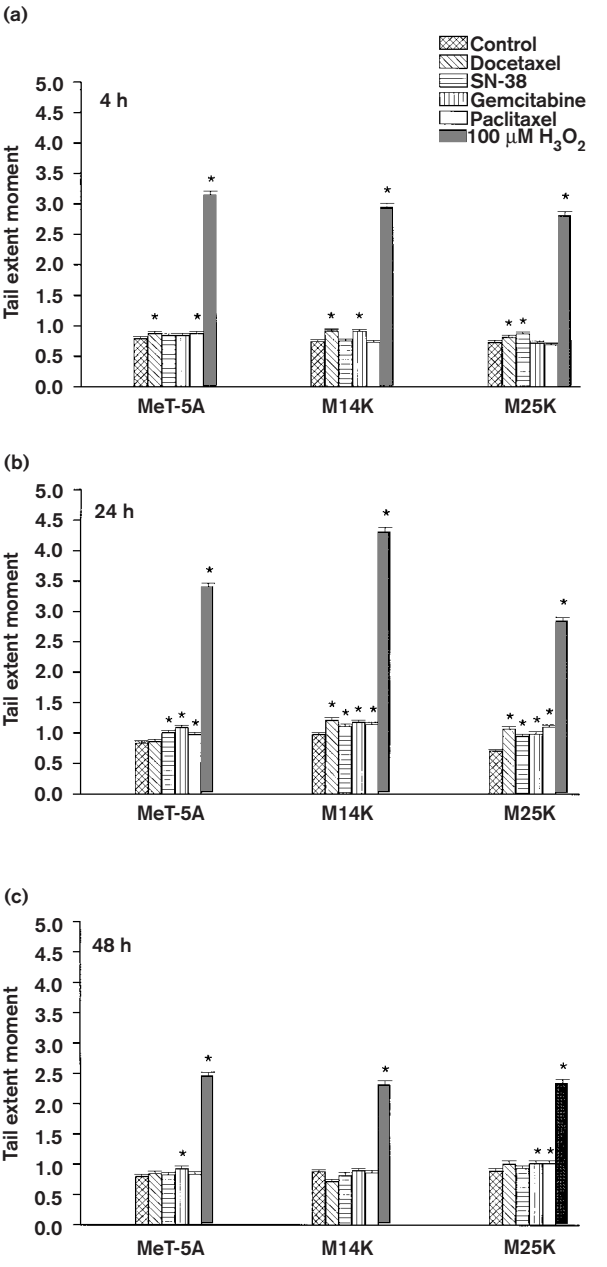

Figure 2. Mean tail extent moment indices for MeT-5A, M14K and M25K cells exposed to 10^{-8} M docetaxel, paclitaxel, SN-38 and gemcitabine for 4 (a), 24 (b) and 48 (c) h. Exposure to 100 μM H_2O_2 for 30 min was used as a positive control. Values are means $\pm$ SEM from two separate experiments. *$p < 0.05$.

the ability of SN-38 to cause DNA damage in mesothelioma cell lines at exposures of up to 24 h. No DNA damage was observed in cells surviving an exposure of 48 h, but the viability of two of the mesothelioma cell lines was low when exposed to 10^{-8} M SN-38. The explanation could be that any damaged cells had undergone apoptosis.

Gemcitabine, a pyrimidine analog of deoxycytidine, prevents the activity of DNA polymerases by becoming incorporated into the end of the elongating DNA strand. Its action is cell cycle specific and it blocks cells at the G_1/S interface. The cytotoxic effects of gemcitabine are schedule dependent and increase with increasing duration of exposure.[17] Our Comet assay results confirmed these observations. Gemcitabine caused significant increases in the tail moment at every exposure time (4, 24 and 48 h) in at least one of the mesothelioma cell lines. However, in the cell viability tests, gemcitabine was the least active agent at a concentration of 10^{-8} M after 48 h exposure. Since gemcitabine action is cell cycle specific, the cells counted in the viability tests could have been in a different phase of the cell cycle and therefore not affected by gemcitabine.

The Comet assay is a sensitive micro-electrophoretic technique for direct visualization of DNA strand breaks in individual eukaryotic cells. DNA SSBs obtained using the highly alkaline Comet assay can be used as an indicator of DNA damage. Compared to other methods, fewer cells are required for the Comet assay. This assay can be readily used as a biomarker of DNA damage because of the sensitivity of the method.[18,19] To our knowledge there are no other studies in which the Comet assay has been used to assess the effects of chemotherapeutic agents on human mesothelioma cell lines. All the agents caused DNA SSBs, even the mitotic spindle poisons, docetaxel and paclitaxel. Exposure for 24 h was crucial. At this exposure time, all four agents caused DNA damage in both mesothelioma cell lines. However, only gemcitabine and paclitaxel caused significant tail moment increases after 48 h exposure and in only one cell line (M25K). The decrease in the tail moment between 24 and 48 h exposures could be explained by DNA repair or by the loss of the most heavily damaged cells.

Conclusion

These *in vitro* testing methods are useful for assessing the sensitivity of mesothelioma cell lines to chemotherapeutic agents. Docetaxel, paclitaxel, SN-38 and gemcitabine were all growth inhibitors of mesothelioma cells, but there were noticable differences in potency between the agents. The cell lines from different patients diverged in their sensitivity to the individual agents, but this could be attributed to their different histological subtypes. It is already known that malignant mesothelioma is a heterogenic disease and this might also cause differences in sensitivity. The sensitivity of the non-malignant cell lines was anticipated, because chemotherapeutic agents are known to be toxic to normal tissues. Our results show that all four chemotherapeutic agents induced DNA SSBs in the two tested mesothelioma cell lines, as well as in the non-malignant (MeT-5A) cells. However, the increases in tail moments recorded were not significantly higher than those caused by the positive control, H_2O_2, which is itself a potent inducer of DNA strand breaks.

We present here two methods for pre-clinical drug evaluation. The cell viability test and the Comet assay are both useful methods for assessing the sensitivity of cell lines to chemotherapeutic agents. These methods would be especially useful for selecting drugs for clinical trials in rare diseases such as mesothelioma. A clinical trial in patients with mesothelioma has been initiated using a combination of docetaxel and SN-38, partly as a result of our *in vitro* studies using the single agents. We plan to investigate other drug combinations using these methods.

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