DNA-AP sites generation by Etoposide in whole blood cells

Emilio Rojas¹, Patricia Mussali¹, Efrain Tovar² and Mahara Valverde*¹

Address: ¹Departamento de Medicina Genómica y Toxicología Ambiental Instituto de Investigaciones Biomédicas. Universidad Nacional Autónoma de México D.F. C.P. 04510, México and ²Centro de Educación Ambiental e Investigación, Sierra de Huautla (CEAMISH), UAEM. Av. Universidad No. 1001, Col. Chamilpa, Cuernavaca, Morelos, CP 62210, México

Email: Emilio Rojas - emilior@servidor.unam.mx; Patricia Mussali - pmussali@yahoo.com.mx; Efrain Tovar - etovar@yahoo.mx; Mahara Valverde* - mahara@biomedicas.unam.mx

*Corresponding author

Published: 16 November 2009
Received: 28 May 2008
Accepted: 16 November 2009

This article is available from: http://www.biomedcentral.com/1471-2407/9/398

© 2009 Rojas et al; licensee BioMed Central Ltd.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Etoposide is currently one of the most commonly used antitumor drugs. The mechanisms of action proposed for its antitumor activity are based mainly on its interaction with topoisomerase II. Etoposide effects in transformed cells have been described previously. The aim of the present study was to evaluate the genotoxic effects of this drug in non-transformed whole blood cells, such as occurs as collateral damage induced by some chemotherapies.

Methods: To determine etoposide genotoxicity, we employed Comet assay in two alkaline versions. To evaluate single cell gel electrophoresis (SCGE) assay experiments revealed etoposide-induced increases in DNA damage in phytohemagglutinin (PHA)-stimulated blood and non-stimulated blood cells. When the assay was performed at a less alkaline pH, 12.3, we observed DNA damage in PHA-stimulated blood cells consistent with the existence of alkali labile sites (ALSs). In an effort to elucidate the molecular events underlying this result, we applied exonuclease III (Exo III) in conjunction with a SCGE assay, enabling detection of DNA-AP sites along the genome. More DNA AP-sites were revealed by Exo III and ALSs were recognized by the SCGE assay only in the non-stimulated blood cells treated with etoposide.

Results: Alkaline (pH > 13) single cell gel electrophoresis (SCGE) assay experiments revealed etoposide-induced increases in DNA damage in phytohemagglutinin (PHA)-stimulated blood and non-stimulated blood cells. When the assay was performed at a less alkaline pH, 12.3, we observed DNA damage in PHA-stimulated blood cells consistent with the existence of alkali labile sites (ALSs). In an effort to elucidate the molecular events underlying this result, we applied exonuclease III (Exo III) in conjunction with a SCGE assay, enabling detection of DNA-AP sites along the genome. More DNA AP-sites were revealed by Exo III and ALSs were recognized by the SCGE assay only in the non-stimulated blood cells treated with etoposide.

Conclusion: Our results indicate that etoposide induces DNA damage specifically at DNA-AP sites in quiescent blood cells. This effect could be involved in the development of secondary malignancies associated with etoposide chemotherapy.

Background

In the last decade, etoposide (also known as VP-16213) has been one of the most commonly used agents for treating a number of malignancies. Etoposide is a semi-synthetic derivative of epipodophyllotoxin derived from the plant Podophyllum peltatum [1-3]. Its primary intracellular target, topoisomerase II, alters DNA topology by passing an intact double helix through a transient double stranded break that it generates in a separate nucleic acid segment [4-6].
Topoisomerase II is required to resolve knots and tangles in the genetic material that are produced by physiological processes such as DNA recombination and replication [7-12]. In the absence of topoisomerase II, cells are unable to segregate daughter chromosomes and die of mitotic failure [13].

In contrast to most drugs that target specific enzymes, etoposide and other topoisomerase II-targeting anticancer agents act in a subtle manner. Rather than blocking the activity of this essential enzyme, etoposide kills cells by increasing the concentration of topoisomerase II-DNA cleavage complexes [7,12,14-16]. This action converts topoisomerase II into a potent cellular toxin that fragments the genome. Consequently, etoposide has been deemed a topoisomerase II poison, distinct from drugs that inhibit the overall catalytic activity of an enzyme [7,12,14-18]. It has been known for more than a decade that etoposide stabilizes topoisomerase II-associated DNA breaks, thereby abolishing the ability of the enzyme to ligate cleaved nucleic acid molecules [7,12,16,19-21]. Specifically when etoposide interacts with topoisomerase IIα, it traps the enzyme in a covalently bound form with its DNA substrate [5,22].

The topoisomerase IIα-DNA complex is stabilized with the etoposide molecule by hydrogen bonds with the nucleic acid bases, and this stabilized complex thus prevents re-ligation of DNA by topoisomerase IIα [23,24]. Both double-and single-strand breaks (SSBs) in DNA can be produced by etoposide.

The production of free radicals during etoposide metabolism has also been observed [25-27]. An orthoquinone metabolite of etoposide can be transformed into a hydroquinone [21]. When oxidized, hydroquinones give rise to hydroxy radicals, which may ultimately contribute to etoposide-associated SSBs in DNA [28]. Although, the etoposide mechanism of action is well described in transformed cells, is important to know the effects generated in non-transformed whole blood cells as they are also exposed to the antineoplastic drug.

The Single Cell Gel Electrophoresis (SCGE) assay, also known as the comet assay, has been proposed as a sensitive, reliable and rapid method for detecting DNA SSBs, alkali labile sites (ALSs), and delayed repair sites (DRSs) in eukaryotic cells under extremely alkaline conditions (pH > 13) [29,30]. Meanwhile, the SCGE assay reveals only SSBs and DRSs under less extreme alkaline conditions (i.e. pH 12.3). Thus by comparing the SCGE results obtained at pH 12.3 to those obtained pH >13, it is possible to discriminate the accumulation of apurinic and apyrimidinic sites (AP sites), which produce ALSs, from other forms of DNA damage.

In this study, we used the alkaline SCGE assay at pH 12.3 and pH >13 in non-stimulated and PHA-stimulated human blood cells to assess the genotoxicity associated with etoposide-induced oxidative stress in non-transformed cells. We performed follow-up assays with exonuclease III enzyme (Exo III) to detect DNA-AP sites within the genome [31]. The effect of co-treatment with an antioxidant, on etoposide genotoxicity was also examined. If etoposide treatment generates the production of reactive oxygen species, principally phenoxy radicals, in non-stimulated whole blood cells, then exposure to an antioxidant should reduce the extent of DNA damage induced.

**Methods**

**Chemical and reagents**

Normal agarose, low melting point agarose (LMPA), ethidium bromide, Tris, Na$_2$EDTA, DMSO (dimethyl sulfoxide), Phytohemaglutinin (PHA), Triton X-100, RPMI-1640 medium, and etoposide were obtained from Sigma Chemical Co. (St. Louis, MO), NaOH and NaCl were obtained from Merck (Mexico) and Baxter (Mexico), respectively. Exo III was obtained from Amersham Life Science (Piscataway, NJ, USA). Ascorbic acid (AA), also known as vitamin C, was obtained from ICN (Mexico).

**Human blood cells and treatments**

The protocol was approved by the Ethics committee of Instituto de Investigaciones Biomédicas at Universidad Nacional Autónoma de México. Whole blood samples were obtained by vein puncture from normal healthy volunteers, who were non-smokers and not taking any medications. PHA-stimulated and non-stimulated whole blood cells were treated for 2 or 24 h with different etoposide concentrations (0, 2.07, 20.7 and 207 μM), in the presence of RPMI-1640 culture medium and maintained at 37°C under 5% CO2 conditions.

The etoposide concentrations were determined by assuming a body surface area of 1.63 m$^2$ for the volunteers and calculating a dose equivalent to that used clinically for hematological malignancies. The calculated equivalent of the clinical dose was taken as the highest concentration applied in our study.

PHA-stimulated whole blood was first incubated for 6 h at 37°C under 5% CO2 conditions in 1 ml of RPMI-1640 culture medium with 71 μl of PHA, and then treated with etoposide as described above. To test the attenuation of etoposide AP-site generation in non-stimulated blood cells, the cultures were treated at the same time with AA (200 μM).

**Viability**

The dual cell-stain assay described by Hartman and Speit [32] was employed to determine the viability of the PHA-
stimulated and the non-stimulated whole blood cells after etoposide treatments. The analysis was performed with a fluorescence microscope (Olympus BX60); 4 fields and at least 400 cells per slide were scored. The results were expressed as percentage of cells alive relative to controls.

**Single cell gel electrophoresis**

The alkaline comet assay was performed essentially as described previously [33]. Briefly, after the experimental treatment was applied, 20-μl samples of whole blood, both PHA-stimulated and non-stimulated, were dissolved in 0.5% LMPA, spread onto microscope slides precoated with 0.5% agarose, and covered with an additional 0.5% LMPA layer. The cells were then lysed in a high salt and detergent solution (2.5 M NaCl, 10 mM EDTA, 10 mM Tris pH 10, with fresh 10% DMSO and 1% Triton x-100), for at least 1 h at 4°C. Subsequently, the cells were placed in a horizontal electrophoresis chamber and exposed to an alkaline solution (300 mM NaOH, 1 mM Na2EDTA, pH >13) for 20 min to allow the DNA to unwind. For DNA electrophoresis, a 25-V electric current (300 mA, 0.8 V/cm) was applied for 20 min. All technical steps were conducted under very dim indirect light. After electrophoresis, the slides were gently removed and the alkaline pH was neutralized by application of 0.4 M Tris, pH 7.5. The slides were dehydrated in two steps with absolute ethanol for 5 min each. Ethidium bromide (75 μl of a 20 μg/ml solution) was added to each slide and a coverglass was placed on the gel.

We performed the comet assay at pH 12.3 as described in our previous report [33]. The comet assay was also used in combination with Exo III as described by Gedik et al. [34]. Briefly, after the experimental treatment was applied, 20-μl samples of whole blood, both PHA-stimulated and non-stimulated, were dissolved in 0.5% LMPA, spread onto microscope slides precoated with 0.5% agarose, and covered with an additional 0.5% LMPA layer. The cells were then lysed in a high salt and detergent solution (2.5 M NaCl, 10 mM EDTA, 10 mM Tris pH 10, with fresh 10% DMSO and 1% Triton x-100), for at least 1 h at 4°C. Subsequently, the cells were placed in a horizontal electrophoresis chamber and exposed to an alkaline solution (300 mM NaOH, 1 mM Na2EDTA, pH >13) for 20 min to allow the DNA to unwind. For DNA electrophoresis, a 25-V electric current (300 mA, 0.8 V/cm) was applied for 20 min. All technical steps were conducted under very dim indirect light. After electrophoresis, the slides were gently removed and the alkaline pH was neutralized by application of 0.4 M Tris, pH 7.5. The slides were dehydrated in two steps with absolute ethanol for 5 min each. Ethidium bromide (75 μl of a 20 μg/ml solution) was added to each slide and a coverglass was placed on the gel.

The measures of AP sites were obtained by subtraction of the mean comet assay score with enzyme buffer alone from that with ExoIII. DNA migration was analyzed on an Olympus BMX60 microscope with epifluorescence equipment (with a 515-560-nm excitation filter and a 590-nm barrier filter). DNA migration measurements (tail image length, in microns) were made with a scaled ocular. To identify the tail, the head of the comet was defined as the most brilliant circular region in the image. One hundred cells were scored for each treatment condition. All experiments were conducted in triplicate and scored in a double blind manner.

**Statistical analysis**

All statistical analyses were performed with STATISTICA software version 5 from STAT Soft Inc. USA (1996). The Mann-Whitney U test was used to determine DNA damage statistical differences between control cells and those treated with etoposide. Student’s t test was used to compare cell viability, and Exo III recognition sites between control and etoposide-treated cells. For the evaluation of ALS, we used the Shapiro-Wilk “W” test which is used to probe normality [35]. We compared each treatment with its particular control (2 h non-stimulated, 2 h stimulated, 24 h non-stimulated and 24 h stimulated) and with each etoposide concentration (0.0, 2.07, 20.7, 207.0). The results reported show that W test was not significant in all case (2 h non-stimulated, W = 0.867, P = 0.06148; 24 h non-stimulated, W = 0.9093, P = 0.20902); 2 h stimulated, W = 0.91198, P = 0.22618; 24 h stimulated, W = 0.93917, P = 0.48735, then the hypothesis that the respective distribution is normal was accepted. We performed a one way ANOVA test in order to detect if there was an effect of the etoposide concentration (0.0, 2.07, 20.7, 207.0) on the formation of akali-labile sites (ALS) in stimulated and non-stimulated cells at 2 h and 24 h.

Thereafter, a multiple comparison test (Tukey) was used to determine the significant differences between group means, particularly we analyzed if the control differed significantly with each etoposide concentration [35]. The relationship between ALS index and net enzyme recognition sites was analyzed by Pearson’s correlation.

**Results**

**Viability**

Our observations of the cells following the dual cell-stain method for PHA-stimulated and non-stimulated whole blood cells revealed that cell viability was high after etoposide treatment for 2 h and 24 h. As shown in table 1, we observed cell viability rates that exceeded 70%.

**Single cell gel electrophoresis**

SCGE at pH >13, which reveals SSBs, DRSs and ALSs, revealed a dose-dependent effect of etoposide treatment (2.07, 20.7 and 207.0 μM) on cells treated for 2 h and 24 h (Figure 1A, B respectively). We observed an increase in DNA-damage in all etoposide treatments showing the highest effect at 207 μM with respect to the control. However, SCGE assay performed at pH 12.3, which detect SSBs and DRSs, but not ALSs, showed genotoxic effects in the PHA-stimulated whole blood cells only at both treatment durations (Figure 2). These results demonstrate oxidative DNA damage generated by etoposide in non-stimulated blood cells, among others sources of ALS generation [35].
Alkali labile site (ALS) index

The ALS index was determined as the difference between the DNA damage detected at pH 12.3 and the damage estimated at pH>13 by the SCGE assay. All data were normalized with respect to the controls. As shown in Figure 3, non-stimulated whole blood cells had higher ALS index values than PHA-stimulated cells treated with etoposide. Control cells differed from etoposide-treated cells in all conditions except in PHA-stimulated cells treated for 24 h. The ANOVA analysis showed that there was a significant effect of etoposide concentration (0.0, 2.07, 20.7, 207.0) on the formation of ALS in all treatments: 2 h non-stimulated (F3 = 442.97, P < 0.0000), 24 h non-stimulated (F3 = 246.66, P < 0.0000) 2 h stimulated (F3 = 50.35, P < 0.0000) 24 h stimulated (F3 = 9.52, P < 0.01); these findings were consistent with the SCGE data (Figure 3).

DNA AP-sites detection

To test the hypothesis that ALSs generated by etoposide-oxidative stress could develop into DNA-AP sites, we used the enzyme Exo III, which recognizes this kind of DNA lesions. This analysis was performed in non-stimulated whole blood cells treated for 2 h with etoposide, a condition which produces a high rate of ALS induction, and the results were compared with the data from the PHA-stimulated whole blood cells exposed to etoposide for 24 h. As shown in figure 4, we observed a relationship between percentage of AP-sites and etoposide concentration in non-stimulated whole blood cells relative to PHA-stimulated blood cells subjected to the longer treatment.

Because the presence of ALSs in non-stimulated whole blood cells was inferred indirectly by comparing the SCGE assays under the two pH conditions, it was important to test whether these putative ALSs involved AP-sites. Therefore we examined whether there was an association between the ALS index data and the DNA AP-sites data, as revealed by the use of Exo III. Indeed, we found a significant positive correlation (r = 0.90; p < 0.01) between ALS index value and the percent of DNA-AP sites detected by Exo-III (Figure 5).

If etoposide treatment induces ROS, principally phenoxyl radicals in non-stimulated whole blood cells, then an antioxidant exposure should reduce the DNA damage induced by etoposide. As shown in figure 6, we found that a 2-h treatment course with the antioxidant AA (200 μM) concurrent with the 2-h etoposide treatment reduced the DNA damage induced by etoposide in non-stimulated whole blood cells. This finding indicates that AA provided some level of protection for the non-stimulated blood cell DNA in the oxidative micro-environment generated by the etoposide.

Discussion

Etoposide affects chromatin function by directly and physically interfering with topoisomerase IIα enzyme activity. Topoisomerase IIα is considered to be an important player in the maintenance of the DNA double helix, due to its capacity to regulate conformational changes in DNA, in normal processes such as replication, transcription or condensation and segregation of chromosomes [4-6].

Topoisomerase IIα activity fluctuates with the cell cycle; its levels elevate as the cells progress through the cycle toward mitosis [36-39]. Thus as structural maintenance of DNA is most challenged during DNA replication, it is expected that cycling cells would be the most susceptible to damage in the presence of etoposide. Because non-stimulated cells are not cycling, they have relatively low Topoisomerase II activity compared to PHA-stimulated whole blood cells, and thus would be expected to be relatively insensitive to DNA-damaging effects of etoposide.

The present findings of etoposide-induced DNA damage in non-stimulated cells differ from the findings of Olive and Banath [40] which indicated an absence of DNA dam-

| Etoposide | 2 h treatment non-stimulated blood cells | 2 h treatment PHA-stimulated blood cells |
|-----------|-----------------------------------------|-----------------------------------------|
| 0 μM      | 100 ± 5.5                               | 100 ± 5.6                               |
| 2.07 μM   | 95.5 ± 8.5                              | 88.6 ± 3.9                              |
| 20.7 μM   | 99.2 ± 2.2                              | 83.2 ± 8.2                              |
| 207 μM    | 91.1 ± 8.2                              | 75.6 ± 7.6                              |

| Etoposide | 24 h treatment non-stimulated blood cells | 24 h treatment PHA-stimulated blood cells |
|-----------|-----------------------------------------|-----------------------------------------|
| 0 μM      | 100 ± 2.3                                | 100 ± 3.9                               |
| 2.07 μM   | 100 ± 3.7                                | 96.7 ± 3.4                              |
| 20.7 μM   | 100 ± 8.0                                | 96.7 ± 3.4                              |
| 207 μM    | 93.8 ± 8.2                               | 81.4 ± 9.9                              |

Viability % ± SD. Each cell represents the average of three independent experiments.
age induction by etoposide in non-cycling cells. This discrepancy is most likely due to inherent differences between the cells used in the experiments. Olive and Banath used colon carcinoma cells (WiDr), while we used human lymphocytes from healthy donors. Lymphocytes, such as those used here, are normally arrested in the G0 phase and non-transformed.

To assess the molecular processes involved in the etoposide treatment-induced breaks, we compared the non-stimulated whole blood cells genotoxicity data generated by the SCGE assay under both highly basic (pH = 12.3) and extremely basic (> 13) pH conditions and thus generated the ALS index values (see results section). This comparison enables the presence of ALSs to be deduced indirectly because the oxidative response capable of generating apurinic or apyrimidic sites (AP sites) is pH-dependent [41,42]. The ALS index data indicated that ALSs, which can be generated by an oxidative stress [6,33], constituted the primary form of DNA damage induced by etoposide in whole blood cells.

To assess whether oxidative stress was responsible for the DNA damage observed in the etoposide-treated non-stim-
Percentage of ALS relative to controls after 2-h and 24-h of etoposide treatment for both non-stimulated and PHA stimulated blood cells. Data were analyzed using one way Anova test. (* p < 0.05 vs. control; ** p < 0.001 vs. control).

Percentage of DNA-AP sites relative to controls evidenced by Exo III in non-stimulated blood cells treated for 2-h and PHA-stimulated blood cells treated 24-h with etoposide. Data were analyzed using one way Anova test. (***p < 0.01 vs. control; ****p < 0.001 vs. control).
Figure 5
Pearson’s correlational analysis between the percent of DNA-AP sites as evidenced by Exo III and ALS index in non-stimulated blood cells treated for 2 h and PHA-stimulated blood cells treated for 24 h with etoposide.

Figure 6
DNA damage induced by etoposide (0, 2.07, 20.7 or 207 μM) in non-stimulated blood cells treated for 2-h (open bars) and DNA damage inhibition produced by AA in non-stimulated blood cells treated for 2 h with etoposide (solid bars). Every bar represents the mean value of three independent experiments. Data were analyzed using the Mann-Whitney U test. (*= p < 0.05; **= p < 0.005 vs. control; ***= p < 0.001 vs. corresponding no AA condition).
ulated cells, we used the SCGE assay in combination with Exo III, an enzyme that recognizes AP sites in DNA. The results corroborated the presence of DNA-AP sites in the genomes of the non-stimulated cells treated with etoposide. Moreover, when non-stimulated cells were exposed to etoposide in the presence of the antioxidant AA, less DNA damage was observed. Thus the ability of the antioxidant to protect the DNA suggests that phenoxyl radicals are the major radicals involved in the oxidative DNA-damage induced by etoposide, especially in non-stimulated cells [27,43, and [44]].

Interestingly, our results suggest that the molecular events by which DNA breaks are generated in PHA-stimulated and non-stimulated cells are quite different. The damage observed in the PHA-stimulated whole blood cells could be explained by the classical events ascribed to topoisomerase II poisons [12].

These have two principal components: DNA strand breaks due to the inhibition of topoisomerase II by etoposide and etoposide-quinone free radical effects [25,45]. Tonnov and colleagues [46] observed DNA damage in leukocytes evidenced by SCGE assay at pH 13, suggesting that etoposide might cause oxidative damage in leukocytes by a mechanism involving inhibition of the enzyme topoisomerase IIβ. Although the exact function of this enzyme has not been resolved, it is known that its concentration is generally independent of cell cycle and cell growth [[12,37,39], and [46]]. In addition, topoisomerase IIβ is expressed at a higher level than topoisomerase IIα in human peripheral blood cells [47]. If the AP site damage observed in the present study was dependent upon an interaction with this enzyme, we should have also observed this kind of damage in PHA-stimulated whole blood cells. However our findings were not consistent with this prediction.

It is our view that the induction of damage at DNA-AP sites observed in non-stimulated whole blood cells herein was due to interaction of the drug metabolites with cellular targets beyond topoisomerase IIβ (which was not present at high levels), such as DNA and/or proteins. Interaction of the drug metabolites with these alternative targets can cause DNA damage by the generation of free radicals [28]. Another possible explanation could be that DNA repair status in non-stimulated whole blood cells are less active than stimulated whole blood cells to remove this kind of DNA damage [48]. Moreover, the reduced DNA damage in the presence of the antioxidant AA suggests that etoposide-hydroquinone phenoxyl radical is the responsible mediator of these effects [27,49].

Conclusion
In summary, our data show that etoposide can produce differential forms of DNA damage in PHA-stimulated and non-stimulated blood cells. These results could have important implications for elucidating the mechanisms associated with the development of secondary malignancies (principally acute myelocytic leukemia) that are associated with the use of etoposide as an antineoplastic drug [50].

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ER was the project leader and directed the study; PM participated in data acquisition and generated experimental data. ET performs the statistical analysis of the data. MV was the main investigator of the study, performed the experimental design and the manuscript draft. She is the corresponding author. All authors reviewed and approved the final manuscript.

Acknowledgements
The authors thank Maria del Carmen López for her technical support. A professional scientific editor at Write Science Right was consulted during the preparation of this manuscript.

References
1. van Maanen JMS, Retel J, de Vries J, Pinedo HM: Mechanisms of action of antitumor drug etoposide: a review. J Natl Cancer Inst 1998, 80:1526-1533.
2. Hainsworth JD, Greco FA: Etoposide: Twenty years later. Annals Oncol 1995, 6:325-341.
3. Hande KR: Clinical Oncology Update, Etoposide: Four decades of development of a topoisomerase II inhibitor. Eur J Cancer 1998, 34(10):1514-1521.
4. Markovits J, Pommier Y, Kerrigan D, Covey JM, Titchen EJ, Kohn KW: Topoisomerase II-mediated DNA breaks and cytotoxicity in relation to cell proliferation and in the cell cycle in NIH-3T3 fibroblasts and L1210 leukemia cells. Cancer Res 1987, 47:2050-2055.
5. McPherson JP, Goldenberg GJ: Induction of apoptosis by deregulated expression of DNA topoisomerase III. Can Res 1998, 58:4519-4524.
6. Boos G, Stopper H: Genotoxicity of several clinically used topoisomerase II inhibitors. Toxical Lett 2000, 116:7-16.
7. Burden DA, Osheroff N: Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. Biochim Biophys Acta 1998, 1400:139-154.
8. Wang JC: DNA topoisomerases. Ann Rev Biochem 1996, 65:633-692.
9. Wang JC: Moving one DNA double helix through another by a type II DNA topoisomerase: The story of a simple molecular machine. Quart Rev Biophys 1998, 31:107-144.
10. Wang JC: Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol 2002, 3:430-440.
11. Nattis JL: Investigating the biological functions of DNA topoisomerases in eukaryotic cells. Biochim Biophys Acta 1998, 1400(1-3):63-81.
12. Fortune JM, Osheroff N: Topoisomerase II as a target for anticancer drugs: When enzymes stop being nice. Prog Nucleic Acid Res Mol Biol 2000, 64:221-253.
13. Bromber KD, Burgin AB, Osheroff N: A two drug model for etoposide action against human topoisomerase II. J Biol Chem 2002, 277:31201-31206.
14. Pommier Y, Feser MR, Goldwasser F: Cancer chemotherapy and Biotherapy: Principles and practice. 2nd edition. Edited by: Chabner BA, Longo DL. Lippincott-Raven Publishers, Philadelphia; 1996:435-461.

15. Li TK, Liu LF: Tumor cell death induced by topoisomerase-targeting drugs. Annu Rev Pharmacol Toxicol 2001, 41:53-77.

16. Wiltermann AM, Osheroff N: Base excision repair intermediates as topoisomerase II poisons. J Biol Chem 2001, 276:17727-17731.

17. Kreuzer KN, Cozarrelli NR: Escherichia coli mutants morpho-sensitive for deoxyribonucleic acid gyrase subunit A: effects on deoxyribonucleic acid replication, transcription, and bacteriophage growth. J Bacteriol 1979, 140:424-435.

18. Andoh T, Ishida R: Catalytic inhibitors of DNA topoisomerase II. Biochim Biophys Acta 1998, 1400:155-171.

19. Osheroff N: Effect of antineoplastic agents on the DNA cleavage/re religation reaction of the eukaryotic topoisomerase II. Biochemistry 1989, 28:6157-6160.

20. Robinson MJ, Osheroff N: Effects of antineoplastic drugs on the poststrand- passage DNA cleavage/re religation equilibrium of topoisomerase II. Biochemistry 1991, 30:1807-1813.

21. Long BH: Mechanisms of action of teniposide (VM-26) and comparison with etoposide (VP-16). Seminars in Oncology 1992, 19:3-19.

22. Kingma P, Osheroff N: The response of eukaryotic topoisomerase II to DNA damage. Biochim Biophys Acta 1998, 1400:223-232.

23. Wang JC: DNA topoisomerases. Annu Rev Biochem 1995, 64:665-697.

24. Pratt WB, Rudden RW, Ensminger WD, Maybaum J: The anticancer drugs. Oxford University Press, New York; 1996.

25. van Maanen JM, Laffeur MV, Mans DR, Aker E van den, Ruiter C, Koepstra PR, Popple D, Van Vries J, Retel J, Pinedo HM: Semiquinone formation from the catechol and ortho-quinone metabolites of the antitumor agent VP-16-213. Free Radic Res Commun 1998, 4(6):371-84.

26. Kalyanaraman B, Nemec J, Sinha BK: Characterization of free radicals produced during oxidation of etoposide (VP-16) and its catechol and quinone derivatives. An Esr study. Biochemistry 1989, 30:4839-46.

27. Kagan VE, Yalowich JC, Day BW, Goldman R, Gantchev TG, Stoyanovsky DA: Ascorbate is the primary reductant of the phenolic antioxidants of topoisomerase II in resting and proliferating human lymphocytes. Biochemistry 2000, 39:3651-60.

28. Wallis SAS, Zhou R, Lilemark E: DNA damage induced by etoposide: a comparison of two different methods for determination of strand breaks in DNA. Cancer Lett 1996, 105:153-159.

29. Rojas E, Lopez MC, Valverde M: Single cell gel electrophoresis assay: methodology and applications. J Chromatogr B 1999, 722:225-254.

30. Tice R, Agurell E, Longo D, Burlinson B, Osheroff N: Base excision repair intermediates as topoisomerase II poisons. J Biol Chem 2001, 276:17727-17731.

31. Herzog CE, Holmes KA, Russchong LM, Genapathy R, Zellweger LA: Absence of topoisomerase II in an asamcraicine-resistant human leukemia cell line with mutant topoisomerase II alpha. Canc Res 1998, 58:5298-5300.

32. Negri C, Chiesa R, Cerino A, Bestagno M, Sala C, Zini N, Maraldi NM, Arcadeli R: Monoclonal antibodies to human DNA topoisomerase I and the two isoforms of DNA topoisomerase II: 170 and 180 kDa isozymes. Exp Cell Res 1992, 200:452-459.

33. Stacey DW, Hitomi M, Chen G: Influence of cell cycle and one gene activity upon topoisomerase II: expression and drug toxicity. Mol Cell Biol 2000, 20:9127-9137.

34. Olive PL, Banath JP: Growth fraction measured using the comet assay. Cell Prolif 1992, 25:447-457.

35. Fortini P, Raspaglio G, Falchi M, Dogliotti E: Analysis of DNA alkylation damage and repair in mammalian cells by the comet assay. Mutagenesis 1996, 11:69-75.

36. Horvatόva E, Slamovόn D, Hlinicovό K, Kumar-Mandal T, Gábelovό A, Collins AR: The nature and origin of DNA single-stranded breaks determined with the comet assay. Mut Res 1998, 409:163-171.

37. Ritov VB, Goldman R, Stoyanovsky DA, Menshikova EV, Kagan VE: Antioxidant paradoxes of phenolic compounds: peroxyl radical scavenger and lipid antioxidant, etoposide, inhibits sarcoplasmic reticulum Ca(2+)-ATPase via thiol oxidation by its phenoxyl radical. Arch Biochim Biophys 1995, 321:140-52.

38. Tsuriya YY, Tsuruva YN, Yalowich JC, Quinn PJ, Claycamp HG, Schnorr NF, Pitt BR, Kagan VE: Phenoxy radicals of etoposide (VP-16) can directly oxidize intracellular thiols: protective versus damaging effects of phenolic antioxidants. Toxicol Appl Pharma- cocol 1995, 131:277-88.

39. Gantchev TG, van Lier JE, Stoyanovsky DA, Yalowich JC, Kagan VE: Interactions of phenoxyl radical of antitumor drug, etopo- side, with reductants in solution and in cell and nuclear homogenates: electron spins resonance and high-performance liquid chromatography. Methods in Enzymology 1994, 234:631-643.

40. Tornov VA, Konoplyannikov MA, Nikolayska TA, Konstantinov EM: Apoptosis of unstimulated human lymphocytes and DNA strand breaks in topoisomerase II inhibitor etoposide (VP16). Biochemistry 1997, 64:345-352.

41. Prosperi E, Negri C, Marchese G, Ricotti GC: Expression of the 170-kDa and 180-kDa isoforms of DNA topoisomerase II in resting and proliferating human lymphocytes. Cell Prolif 1994, 27:257-267.

42. Nospukel T, Hanawa LC, DNA repair in terminally differenciated cells. DNA repair 2002, 1:59-75.

43. Wenzel U, Nickiel A, Kunz S, Daniel H: Ascorbic acid suppresses drug-induced apoptosis in human colon cancer cells by scavenging mitochondiral superoxide anions. Cancerogenesis 2004, 25(5):703-712.

44. Liu WM, Ocklery PR, Joe SP: Exposure to low concentrations of etoposide reduces the apoptotic capability of leukaemic cell lines. Leukemia 2002, 16:1705-1712.

Pre-publication history
The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/9/398/prepub