Pergamon

Biochemical Pharmacology, Vol. 47, No. 9, pp. 1531–1535, 1994.
Elsevier Science Ltd
Printed in Great Britain.

0006-2952(94)E0039-N

SELENITE INDUCTION OF DNA STRAND BREAKS AND APOPTOSIS IN MOUSE LEUKEMIC L1210 CELLS

JUNXUAN LU,* MARK KAECK, CHENG JIANG, ADRIAN C. WILSON and HENRY J. THOMPSON

Division of Laboratory Research, AMC Cancer Research Center, Denver, CO 80214, U.S.A.

(*Received* 13 *October* 1993; *accepted* 14 *December* 1993)

Abstract—The effects of selenite on DNA integrity, cell viability, and long-term proliferative potential of mouse leukemic L1210 cells were examined in this study. Selenite treatment resulted in concentration-dependent increases in DNA single-strand breaks and double-strand breaks, as detected by a modified filter elution assay. A time–course experiment showed that DNA single-strand breaks preceded DNA double-strand breaks. Agarose gel electrophoresis of DNA extracted from selenite-treated cells displayed a nucleosomal fragmentation pattern that is characteristic of apoptotic cell death. The involvement of a Ca^{2+},Mg^{2+}-dependent endonuclease responsible for DNA double-strand fragmentation was implied by the observation that two inhibitors of endonuclease activity, i.e. aurintricarboxylic acid and zinc, blocked selenite-induced DNA double-strand breaks. These inhibitors also prevented selenite-induced cell death as defined by loss of ability to exclude trypan blue dye. Selenite treatment severely impaired the colony-forming ability of cells capable of trypan blue exclusion. The induction of DNA strand breaks and commitment to apoptosis may explain the selenite-mediated growth inhibition and loss of long-term proliferative potential.

Key words: selenium; DNA strand breaks; apoptosis; leukemic cells

The results of over 50 laboratory studies indicate that ingestion of pharmacological amounts of selenium inhibits the occurrence of chemically or virally induced tumors in several organ systems (reviewed in Refs. 1–4). However, the lack of knowledge about how selenium exerts its anti-carcinogenic activity and concerns about the narrow dose range over which the element is well tolerated have limited the consideration of selenium in clinical use.

Much of what is known about the "mechanisms" of the cancer inhibitory activity of selenium is derived from cell culture experiments. Exposure to various forms of selenium at concentrations between 2 and 5 μM has been observed to inhibit macromolecular synthesis and cell growth without affecting cell viability [5]. These *in vitro* results have been interpreted to indicate that selenite exerts an anti-proliferative effect, which might account, at least in part, for the anticancer activity of the element *in vivo* [2].

In the present study, the effects of selenite on DNA integrity and colony-forming ability (i.e. long-term proliferation potential) of mouse leukemic L1210 cells were investigated. Selenite was chosen as a model selenium compound because its cancer inhibitory activity has been widely studied. Our results show that selenite treatment induced DNA SSBs†, followed by the activation of endonucleases that produced DNA DSBs and the induction of cell death by apoptosis. The induction of DNA SSBs and the activation of the apoptotic cell death pathway might explain the observed severe loss of long-term proliferative potential of selenite-treated cells. Although the effect of selenite on DNA integrity has been noted previously [6, 7], selenium-induced apoptotic cell death has not been studied extensively. Apoptosis occurs when a cell initiates a cascade of biochemical and morphologic events resulting in active death [8, 9] and is inducible in response to treatment by both physiologic and pharmacologic agents [10]. Selenium-induced apoptosis may underlie its cancer inhibitory activity.

MATERIALS AND METHODS

Cell culture. Mouse L1210 leukemia cells were grown in suspension culture in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) and 1 mM glutamine at 37° in a 5% CO_2/95% air atmosphere. Stock cells were maintained at densities $<5 \times 10^5$ cells/mL to minimize spontaneous nucleosomal DNA fragmentation and apoptosis [11]. Cells were seeded at 10^5 cells/mL, and treatment with selenite and/or endonuclease inhibitors was initiated at that time. A selenite stock solution (J. T. Baker Inc., Phillipsburg, NJ) was diluted with complete RPMI 1640 medium and added to the cell suspension to give the required final concentration.

Cell viability. After selenite exposure, cells were counted using a hemocytometer. Cells able to exclude trypan blue after a 5-min incubation with the dye were considered viable.

* Corresponding author: Dr. J. Lu, Division of Laboratory Research, AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214. Tel. (303) 239-3348; FAX (303) 233-9562.

† Abbreviations: SSBs, single-strand breaks; DSBs, double-strand breaks; AE, alkaline elution; NE, neutral elution; and ATA, aurintricarboxylic acid.

Colony formation assay. The colony-forming ability of trypan blue-excluding cells was assessed by their growth in soft agar. This assay reflects the long-term proliferative potential of the assessed cell population. The assay was performed in 13 × 100 mm screw cap culture tubes (Falcon No.2027) as follows. After selenite exposure for 24 hr, cells were counted, collected by centrifugation (200 *g* for 5 min), and washed twice with fresh medium. The cells were resuspended in fresh medium at 10^5 viable cells/mL and serially diluted. A total of 1500 dye-excluding cells in 1.5 mL of fresh medium was mixed with 4.5 mL of molten agar (final agar concentration 1 g/L in fresh medium) and then briefly cooled on ice to facilitate solidification. The cells were incubated at 37° for 7–10 days until colonies grew visible for counting.

[^{3}H]-*Thymidine labeling.* Exponentially growing cells were seeded at 10^5 cells/mL and were exposed to 0.1 μCi/mL of [*methyl*-^{3}H]thymidine (20 Ci/mmol, Du Pont–New England Nuclear, Wilmington, DE) for 24 hr. The percentage of ^{3}H-incorporation ranged from 70 to 80%. Cells were recovered by centrifugation at 200 *g* for 5 min and resuspended at 10^5/mL for treatments.

Filter elution analysis of DNA strand breaks. A gravity-flow alkaline elution method [12] was used with modification to yield information on the occurrence of both single- and double-stranded DNA breaks [13]. Collection of the lysis and proteinase K digestion fractions prior to application of the alkaline elution (AE) buffer constitutes a mini-"neutral elution" (NE), which serves as a reasonable estimate of DNA double-strand breaks as recently demonstrated by our laboratory [13]. The elution parameters were:

$$\text{Double-strand breaks as neutral elution} = [N/(N + A + F)] \times 100\%$$

$$\text{Single-strand breaks as alkaline elution} = [A/(A + F)] \times 100\%$$

where N was ^{3}H collected in lysis and proteinase K digestion steps, A was ^{3}H collected in alkaline elution, and F was ^{3}H retained on filter after alkaline elution.

DNA isolation and gel electrophoresis. After a defined treatment period, 3 million cells were recovered by centrifugation at 200 *g* for 5 min at 4°. Cell pellets were lysed in 1 mL of a buffer containing 10 mM Tris–HCl, pH 8.0, 100 mM EDTA, 0.5% SDS and 0.5 mg/mL proteinase K at 37° overnight and extracted twice with phenol:chloroform. Nucleic acids were precipitated with 0.6 vol. of isopropanol in the presence of 0.2 M NaCl. The DNA was resuspended in 30 μL of 10 mM Tris–HCl, 1 mM EDTA, pH 7.5, treated with ribonuclease to digest RNA, loaded into a 1.5% agarose gel containing 0.1 μg/mL of ethidium bromide, and electrophoresed. Gels were photographed with Polaroid film using UV illumination.

Selenium analysis. Cells were harvested via filtration onto glass fiber discs (0.2 μm pore size, Whatman, Maidstone, UK) and washed with phosphate-buffered saline. Filters were transferred to digestion flasks and wet-ashed in a concentrated nitric:perchloric acid mixture (3:1, v/v). Selenium content of the digest was determined fluorometrically as previously described [14].

Statistical analysis. Data were evaluated for their compliance with assumptions of distributional normality and then subjected to analysis of variance with post hoc comparisons among treatment groups according to the method of Tukey as described in Ref. 15. Concentration–response data were evaluated by polynomial regression analyses [15].

RESULTS

Selenite treatment of L1210 cells for 24 hr resulted in a concentration-dependent increase in cellular selenium content (Table 1). Over the concentration range of 0–20 μM selenite, cellular selenium increased approximately 11-fold. The response pattern was best described mathematically by a quadratic function ($P < 0.004$). In cells accumulating these levels of selenium, concentration-dependent reductions in cell viability ($P < 0.05$) and colony-forming ability ($P < 0.05$) were observed. The greatest percent reductions (20 μM selenite vs 0) were a 53% decrease in viability and a 94% decrease in colony-forming ability. It is noteworthy that at the 5 μM exposure

Table 1. Effect of selenite treatment for 24 hr on cellular selenium content, cell viability by trypan blue exclusion, and colony-forming ability of L1210 cells

Selenite (μM)	Cellular selenium*† (μg/mg DNA)	% Dye-excluding cells*	Number of colonies per 1500 dye-excluding cells*‡
0	~0.5^c	98.2 ± 1.1^a	407 ± 23^a
5	0.9 ± 0.5^{bc}	98.2 ± 0.6^a	73 ± 4^b
10	1.9 ± 0.5^b	76.0 ± 3.6^b	54 ± 13^{bc}
20	5.9 ± 1.0^a	45.7 ± 6.0^c	26 ± 7^c

* Values are means ± SEM (N = 3). Means within a column bearing different superscripts were significantly different as determined by the Tukey test, $P < 0.05$.

† L1210 cells were treated with increasing concentrations of selenite for 24 hr. Cellular DNA was measured by a dye-binding method [16]. A significant concentration effect of selenite ($P < 0.01$) was indicated by analysis of variance.

‡ Viable cells were determined by their ability to exclude trypan blue dye. A total of 1500 dye-excluding cells were seeded into soft agar medium for the colony formation assay.

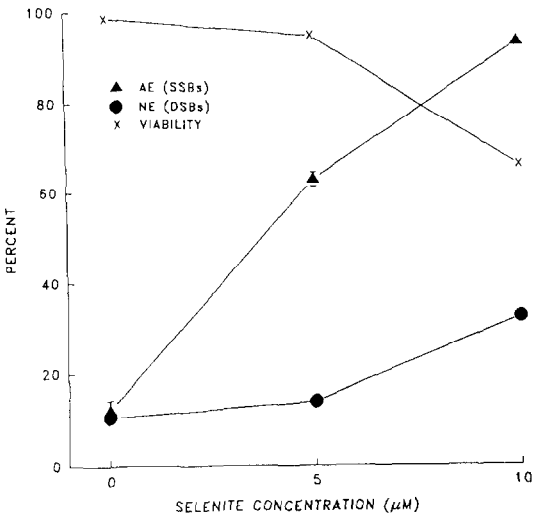

Fig. 1. Concentration-dependent induction by selenite treatment for 24 hr of DNA single-strand breaks (SSBs) as measured by alkaline elution (AE) and DNA double-strand breaks (DSBs) as measured by neutral elution (NE) and loss of trypan blue excluding ability (viability). A representative experiment is shown; each point in this experiment represents the mean of three determinations. The standard error of the mean for most points was within the size of the symbol.

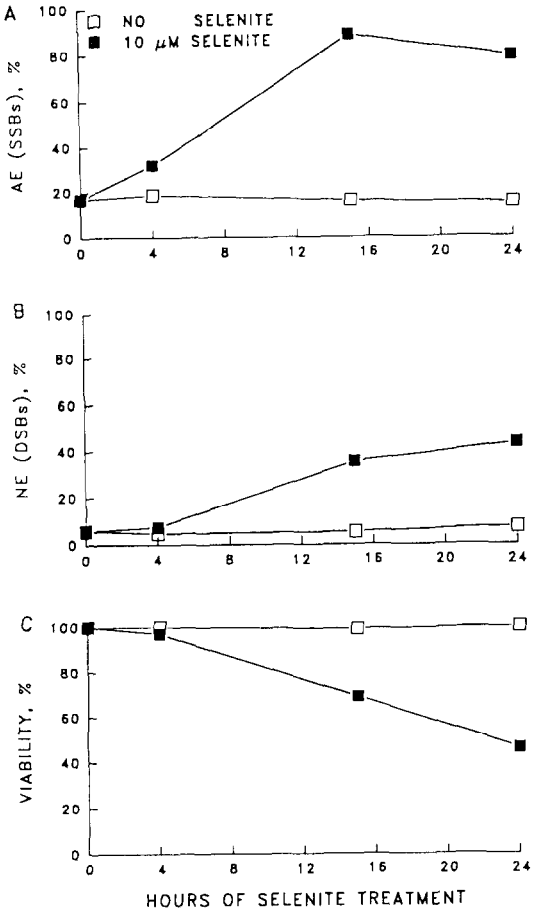

Fig. 2. Time-course of selenite induction of DNA single-strand breaks (SSBs) as measured by alkaline elution (AE, panel A) and DNA double-strand breaks (DSBs) as measured by neutral elution (NE, panel B) and loss of trypan blue excluding ability (panel C). A representative experiment is shown; each point in this experiment represents the mean of three determinations. The standard error of the mean for most points was within the size of the symbol.

level, cell viability was not affected but the colony-forming ability was reduced by 82%.

The effect of selenite treatment for 24 hr on DNA integrity measured by filter elution from a representative experiment is shown in Fig. 1. It was observed that selenite treatment induced concentration-dependent increases in DNA SSBs ($P < 0.001$) and in DNA DSBs ($P < 0.01$). However, the response patterns were very different for the two types of DNA breaks. For SSBs, the relative increase was 415% between 0 and 5 μM selenite and 48% between the 5 and 10 μM levels of exposure. For DSBs, the relative increase was only 33% between 0 and 5 μM exposure levels ($P = 0.05$) and 130% between 5 and 10 μM selenite.

A representative time-course experiment on selenite treatment-induced DNA strand breaks is shown in Fig. 2. It was observed that the increase in SSBs induced by 10 μM selenite preceded the increase in DSBs ($P < 0.05$). The decrease in cell viability observed with increasing duration of selenite treatment more closely paralleled the increase in DSBs than SSBs (Fig. 2C).

Because the concentration–response and time–course results suggest interesting associations between SSBs, DSBs and cell viability, the experiments shown in Table 2 were conducted in an effort to establish the relationships among these events. It was observed that inhibitors of Ca^{2+},Mg^{2+}-dependent endonuclease activity known to be involved in the apoptotic degradation of DNA, i.e. ATA or Zn^{2+} (as $ZnSO_4$), blocked selenite-induced formation of DSBs. This effect was also associated with a 33–41% reduction in SSBs depending on whether zinc or ATA was used as the inhibitor. Of particular interest was the lack of effect of selenite on cell viability when the induction of DSBs was blocked by these inhibitors. Agarose gel electrophoresis of DNA extracted from selenite-treated cells is shown in Fig. 3. A pattern of DNA nucleosomal fragmentation that is typical of apoptosis was observed at 20 μM selenite. We have observed this same type of DNA fragmentation at levels of selenite exposure as low as 5 μM. As shown in Fig. 3, ATA reduced the amount of DNA nucleosomal fragmentation in a concentration-dependent manner.

DISCUSSION

Our finding that selenite treatment of L1210 cells induced DNA SSBs confirms work from our and other laboratories utilizing different methodologies

Table 2. Effects of cotreatments of L1210 cells for 24 hr with selenite and/or inhibitors of endonuclease on DNA strand breaks and cell viability

Treatment	Selenite (μM)	DNA SSBs as alkaline elution* (%)	DNA DSBs as neutral elution* (%)	Viability† (%)
None	0	5.7 ± 0.7^{d}	5.0 ± 0.1^{c}	99
0.25 mM ATA‡	0	4.3 ± 0.4^{d}	3.4 ± 0.2^{c}	99
39 μM $ZnSO_4$	0	6.0 ± 1.1^{d}	5.9 ± 0.1^{bc}	99
Selenite (Se)	10	93.4 ± 0.4^{a}	27.1 ± 1.6^{a}	72
Se + 0.25 mM ATA	10	54.9 ± 0.9^{c}	3.7 ± 0.1^{c}	97
Se + 39 μM $ZnSO_4$	10	62.4 ± 1.9^{b}	8.8 ± 0.6^{b}	95

* Values are means ± SEM (N = 4). Means within a column bearing different superscripts were significantly different ($P < 0.05$).

† Cells able to exclude trypan blue were considered viable.

‡ ATA, aurintricarboxylic acid.

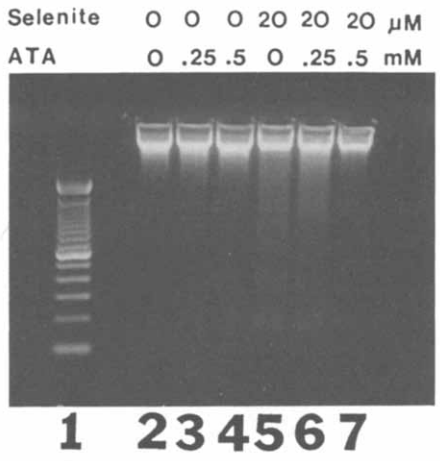

Fig. 3. Agarose gel electrophoretic analysis of DNA isolated from L1210 cells treated for 24 hr with selenite in the absence/presence of aurintricarboxylic acid (ATA). Lane 1, 100-bp DNA size markers; lane 2, untreated control cells; lanes 3 and 4, cells treated with 0.25 and 0.5 mM ATA, respectively; lane 5, cells treated with 20 μM selenite; and lanes 6 and 7, cells cotreated with 20 μM selenite and 0.25 and 0.5 mM ATA, respectively.

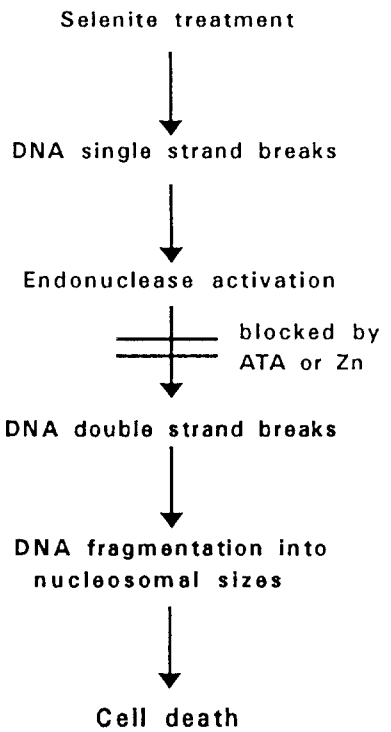

Fig. 4. Proposed sequence of events in selenite-induced apoptotic death of L1210 cells.

and cell types [6, 7, 17]. The present study extends those observations in several ways. Both the concentration–response (Fig. 1) and time–course data (Fig. 2) indicated that the induction of SSBs was associated with the subsequent formation of DSBs and loss of cell viability. Agarose gel analysis of the DNA from selenite-treated cells (Fig. 3) indicated that the DSBs detected by neutral elution were of a type associated with apoptosis. The latter observation was corroborated further by the finding that ATA or zinc, both known inhibitors of endonuclease activity associated with the apoptotic degradation of DNA, blocked both the occurrence of DSBs detected by neutral elution (Table 2) and the ladder-type pattern of DNA fragmentation characteristic of apoptosis (Fig. 3). These observations led us to propose the following working hypothesis about the sequence of events in selenite-induced cell death. Selenite treatment induces DNA SSBs; the accumulation of SSBs triggers endonuclease activation; the endonuclease cleaves DNA as DSBs and the final stage of this cleavage can be detected as oligonucleosomal size fragments on an agarose gel; cells that incurred irreparable DSBs die by apoptosis. This scheme of events is shown in Fig. 4. One question concerning our interpretation of the present data remains (Table 2), that is, cotreatment of L1210 cells with selenite and endonuclease inhibitors blocked DSB formation and

loss of cell viability, but also caused a 31–45% reduction in SSBs. Therefore, it is currently unclear whether the inhibition of endonuclease activity or the reduction of SSBs accounts for blocking the formation of DSBs and the loss of cell viability by these inhibitors. While this issue merits further investigation, either possibility is consistent with our proposed model of selenite-mediated induction of apoptosis. What is clear from the work presented in this study is that the belief that selenite-treated cells die exclusively by the process of necrosis is incorrect. Rather a component of the cell death attributed to treatment with this form of selenium is mediated by apoptosis. Formulation of this hypothesis has merit to ongoing research using forms of selenium that enter at different points in the intermediary pathway of selenium's reductive metabolism and methylation. Given that some of these forms of selenium have anticancer activity and have been reported not to induce SSBs [17], it will be of value to compare their effects on cell death induction to the pathway proposed for selenite in this study.

The observations reported in this study concerning the effect of selenite on cell viability and long-term proliferative potential, in concert with the observation that selenite treatment induces apoptosis may also provide insights that help explain previous reports of the cancer growth inhibitory activity(s) attributed to selenite. For example, it was reported that selenite-treated L1210 cells judged to be metabolically viable by trypan blue exclusion failed to grow or to cause the death of host mice into which these cells were injected, whereas the injection of untreated L1210 cells into mice led to lethality [18]. We suggest that this observation may be explained by selenium-induced commitment of L1210 cells to undergo apoptosis and the loss of long-term proliferative potential. The inability of the L1210 cell population to grow *in vivo* following selenite treatment may be analogous to the effect of selenite exposure on colony formation reported in this study.

Several key questions regarding the mechanism(s) of the biological activity of selenite are raised by the observations reported in this study. They include: (1) are SSBs induced by selenite *per se* or by a selenium species derived from its metabolism; (2) are SSBs induced by a non-selenium mediator that is generated as a secondary consequence of selenite metabolism; (3) do SSBs induced by selenite treatment directly account for the induction of DSBs; (4) does the induction of SSBs by selenite have effects independent of the formation of DSBs; and (5) can DSBs associated with the occurrence of apoptosis be induced by forms of selenium other than selenite? It is hoped that answers to these questions may provide new insights into the mechanism(s) by which selenium inhibits the development of cancer.

Acknowledgements—The authors thank Gretchen Garcia and Mary Witt for technical assistance and Joyce Manley and Connie Ryan for preparation of the manuscript. This work was supported by PHS Grant CA 45145 from the National Cancer Institute to Henry J. Thompson.

REFERENCES

1. Ip C, The chemopreventive role of selenium in carcinogenesis. *J Am Coll Toxicol* **5**: 7–20, 1986.
2. Medina D and Morrison DG, Current ideas on selenium as a chemopreventive agent. *Pathol Immunopathol Res* **7**: 187–199, 1988.
3. Ip C and Medina D, Current concept of selenium and mammary tumorigenesis. In: *Cellular and Molecular Biology of Mammary Cancer* (Eds. Medina D, Kidwell W, Heppner G and Anderson EP), pp. 479–494. Plenum Press, New York, 1987.
4. Shamberger RJ, The genotoxicity of selenium. *Mutat Res* **154**: 29–48, 1985.
5. Morrison DG, Berdan RC, Pauly DF, Turner DS, Oborn CJ and Medina D, Selenium distribution in mammary epithelial cells reveals its possible mechanism of inhibition of cell growth. *Anticancer Res* **8**: 51–63, 1988.
6. Snyder RD, Effects of sodium selenite on DNA and carcinogen-induced DNA repair in human diploid fibroblasts. *Cancer Lett* **34**: 73–81, 1987.
7. Garberg P, Stahl A, Warholm M and Hogberg J, Studies of the role of DNA fragmentation in selenium toxicity. *Biochem Pharmacol* **37**: 3401–3406, 1988.
8. Kerr JFR, Shrinkage necrosis: A distinct mode of cellular death. *J Pathol* **105**: 13–20, 1971.
9. Wyllie AH, Kerr JFR and Currie AR, Cell death: The significance of apoptosis. *Int Rev Cytol* **68**: 251–306, 1980.
10. Eastman A, Apoptosis: A product of programmed and unprogrammed cell death. *Toxicol Appl Pharmacol* **121**: 160–164, 1993.
11. Jiang C, Lu JX, Garcia G and Thompson HJ, Spontaneous nucleosomal DNA fragmentation in a mouse leukemic L1210 cell line. *Biochem Biophys Res Commun* **194**: 836–841, 1993.
12. Hincks JR and Coulombe RA Jr, Gravity-flow alkaline elution: A method to rapidly detect carcinogen-induced DNA strand breaks. *Biochem Biophys Res Commun* **137**: 1006–1014, 1986.
13. Kaeck MR, Briggs S and Thompson HJ, Alkaline elution analysis of DNA fragmentation induced during apoptosis. *Anal Biochem* **208**: 393–396, 1993.
14. Hasunuma R, Ogawa T and Kawanishi Y, Fluorometric determination of selenium in nanogram amounts in biological materials using 2,3-diaminonaphthalene. *Anal Biochem* **126**: 242–245, 1982.
15. Snedecor GW and Cochran WG, *Statistical Methods*, 6th Edn. Iowa State University Press, Ames, IA, 1967.
16. Cesarone CF, Bolognesi C and Santi L, Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal Biochem* **100**: 188–197, 1979.
17. Wilson AC, Thompson HJ, Schedin PJ, Gibson NW and Ganther HE, Effects of methylated forms of selenium on cell viability and induction of DNA strand breakage. *Biochem Pharmacol* **43**: 1137–1141, 1992.
18. Milner JA and Hsu CY, Inhibitory effects of selenium on the growth of L1210 leukemic cells. *Cancer Res* **41**: 1652–1656, 1981.