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Food and Chemical Toxicology 46 (2008) 1619–1626
www.elsevier.com/locate/foodchemtox

Genotoxicity of low dose *N*-nitroso propoxur to human gastric cells

H.H. Kuo, S.S. Shyu, T.C. Wang *

Institute of Cellular and Organismic Biology, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 11529, Taiwan

Received 22 December 2006; accepted 3 January 2008

Abstract

Propoxur is among the most popular insect control agents in subtropical countries such as Taiwan. As a member of the *N*-methylcarbamate insecticide group, propoxur is notorious for its potential for conversion into highly genotoxic *N*-nitroso derivatives. Due to the fact that the stomach has been identified as the major target for *N*-nitroso *N*-methylcarbamates, this investigation used a human gastric cell line, SC-M1, in order to obtain results pertinent to the authentic adverse effects of this compound on human health. This report reveals that at dose levels inhibiting $\leqslant$10% cell growth, a 2-h pulsed treatment of *N*-nitroso propoxur induced significant amounts of DNA damage. Most of the damaged DNA was repaired within 24 h after treatment removal, such that an outcome with a significant induction of chromosomal aberrations was not observed. Gene mutations and anchorage independence, on the other hand, were significantly induced by this same treatment. In conclusion, exposure to low doses of *N*-nitroso propoxur is not cytotoxic nor clastogenic, nevertheless, has the potential to increase genetic instability and, possibly as a result, to enhance the malignant potential of treated cells. We suggest that although the damaged DNA was repaired during the transient G2/M arrest period, it was probably not done in an appropriate way which would preserve the original genetic stability.

Keywords: Carbamate insecticide; Human gastric cell; Malignant potential

1. Introduction

N-Methylcarbamate esters are inhibitors of enzyme cholinesterase in insects. They are insecticides of economic importance, manufactured in very large quantities, and widely used for controlling agricultural and household insect pests. Due to its lower acute toxicity to mammals, *N*-methylcarbamate is gaining popularity over organophosphate insecticides, another cholinesterase inhibitor, especially for the control of insects in household areas, where contamination of the human environment can easily occur. *N*-Methylcarbamate insecticides are converted to *N*-nitroso metabolites by *N*-nitrosation, under conditions of stomach physiology. *N*-Nitroso methylcarbamates are not cholinesterase inhibitors and are less toxic to mammals than the parent *N*-methylcarbamate insecticides. They are nonetheless quite carcinogenic and mutagenic. Previously, we demonstrated their genotoxicities in Chinese hamster V79 lung cells and preneoplastic transformation in primary rat tracheal epithelial cells (Wang et al., 1998a,b). Tumorigenesis experiments in a rodent model indicated that *N*-nitroso *N*-methylcarbamate insecticides mainly target the forestomach (Lijinsky, 1992). In view of this, the results would be more relevant if cells originating from the target tissue could be used for the investigation. Therefore a human gastric cell line, SC-M1, isolated from a local patient in Taiwan was employed in the present study to explore the adverse effects induced by *N*-nitroso propoxur, an *N*-nitroso derivative of a major *N*-methylcarbamate insecticide used in Taiwan.

2. Materials and methods

2.1. Cell culture

SC-M1, a gastric adenocarcinoma cell line, kindly provided by Dr. C-W Chi of Veterans General Hospital, Taipei, Taiwan, was previously established from fresh tumor tissue removed from a 72-year-old

* Corresponding author. Tel.: +886 2 27899540; fax: +886 2 27858059.
E-mail address: tcwang@sinica.edu.tw (T.C. Wang).

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doi:10.1016/j.fct.2008.01.014

male patient at Triservice General Hospital, Taiwan (Tzeng et al., 1991). It was cytogenetically analyzed at the 200th passage, revealing a near-triploid modal karyotype in the cell line.

2.2. N-Nitrosation of propoxur insecticides

Preparation of *N*-nitroso derivatives of propoxur (*O*-isopropoxyphenyl methyl carbamate, CAS 114-26-1) followed the method described by Blevins et al. (1977a,b). Propoxur (98.7%, Chem Service, West Chester, PA) was dissolved in glacial acetic acid (Merck, Darmstadt, Germany), cooled in ice, and added with sodium nitrite (Merck). The solution was kept cold for 1 h and then at room temperature for another hour before being added with ether and ddH_2O. The yellow ether layer was separated off and dried using anhydrous sodium sulfate. The residual *N*-nitroso propoxur was confirmed by checking its maximum intensity at 420, 400, and 385 nm on a Hitachi U2000 spectrophotometer.

2.3. Measurement of cell kinetics

Cells were cultivated in relative low densities as described previously (Kalweit et al., 1999), which allowed the colony size to be measured. Briefly, 1000 cells were plated into each grid of a 60-mm dish. Numbers of individual cells in each colony were counted 24, 44, 48, and 68 h after the initial plating using a dissecting microscope. Based on the distribution of the colony sizes, a proliferation index was derived according to the following formula: Proliferation index $= [(c1 \times 1) + (c2 \times 2) + (c4 \times 3) + (c8 \times 4) + (c16 \times 5)]/n$, where $c1$, $c2$, $c4$, $c8$, and $c16$ each indicates the number of single-cell, 2-cell, 4-cell, 8-cell, and 16-cell colonies, respectively, and n is the total number of cell colonies.

2.4. Measurement of growth inhibition

The method using UV absorption as an approximation for the cell number proposed by Chang (1991) was used to measure the growth inhibition induced by *N*-nitroso propoxur. Briefly, 3×10^5 SC-M1 cells were plated onto each 60-mm Petri dish for overnight. After treatment with *N*-nitroso propoxur in a series of different doses for 2 h, cells were trypsinized and collected in a centrifuge tube, washed twice with cold calcium magnesium-free phosphate-buffered saline (CMF-PBS), extracted twice with cold methanol: acetic acid (3: 1) to remove free nucleosides and nucleotides, and dissolved at 37 °C in a 0.2 N NaOH solution at 10^6 cells/ml for 24 h. The absorption at 260 nm for each cell solution was determined using a Hitachi U2000 spectrophotometer. The percent growth inhibition of each treatment was estimated by dividing the absorbance of the treatment by that of the control.

2.5. Apoptosis assay

After treatment removal and at the indicated times, 0.5 ml of approximately 1×10^6 cells/ml were supplemented with 10 μl Media Bonding Reagent and 1.25 μl Annexin-V-FITC (Apoptosis Detection Kit, Calbiochem®). The mixture was then incubated for 15 min at room temperature in the dark and then centrifuged at 1000*g* for 5 min. The cell pellet was re-suspended in 0.5 ml cold 1X binding buffer. Immediately before analysis with a Coulter Epics XL-MCL flow cytometer, 10 μl PI was added to the suspension. Experiments were performed in triplicate.

2.6. Single-cell alkaline electrophoresis (comet assay)

Fifty millilitre of a cell suspension containing 2×10^5 cells in PBS and 250 μl of an agarose solution were thoroughly mixed, and 65 μl of the mixture was pipetted onto the slide pre-coated with 1% normal-melting point agarose. Seventy millilitre of 1% low-melting point agarose was applied as the third layer of agarose. The slides were immersed in an ice-cold lysis solution (2.5 M NaCl, 100 mM sodium ethylenediaminetetraacetic acid (EDTA), and 10 mM Tris, with the pH adjusted to 10.0 with NaOH; 1% *N*-lauroylsarcosine, 1% Triton X-100, and 10% dimethyl sulfoxide were added immediately before use) at 4 °C overnight. Slides were then denatured in an electrophoresis tank containing 0.3 M NaOH (pH 13.4) and 1 mM sodium EDTA for 20 min. Electrophoresis was carried out at 25 V, 300 mA for 30 min. Slides were washed, blotted, and then transferred to 0.4 M Tris–HCl at pH 7.5 before being stained with YOYO-1 (Molecular Probes; 50 μl of a 20 μM solution prepared in glycerol/Na_2EDTA 0.4 M at pH 7.4 (50/50), containing 0.1% 8-hydroxyquinoline). The COMET image of 100 cells per treatment was recorded with a fluorescence microscope (shortwave pass filter 450–490 nm, chromatic beam splitter 510 nm, and longwave pass filter 520 nm) and a digital camera (DCS-420; Kodak, Rochester, NY, USA). Migration of DNA from the nucleus in each cell was expressed by the parameter of the tail moment and measured with Comet Assay III software (available from www.perceptive.co.uk).

2.7. Flow cytometry for γH2AX

The cell preparation for γH2AX staining followed that described by Olive (2004). Treated cells were fixed in 70% ethanol overnight, and then were permeabilized and rehydrated in a cold TST solution (0.25% Triton X-100 and 4% FBS in PBS) for 15 min. Samples were centrifuged, and the pellet was resuspended in 100 μl mouse monoclonal anti-γH2AX (Upstate Biotechnology) diluted 1:500 in TST at room temperature for 3 h. At the end of incubation, cells were washed by repeatedly centrifuging and resuspending them in 3–4 ml PBST (0.05% Tween 20 in PBS). The cell pellet then was resuspended in 50 μl anti-mouse-IgG-FITC (Jackson Immuno Research) for 30 min at room temperature before being washed and centrifuged in PBST. The cell pellet was further resuspended in a PI solution (containing 0.2 ml of 20 μg/ml PI, 0.2 ml of 5% Triton X-100, 0.1 ml of 2 mg/ml RNase A, and 0.5 ml PBS).

2.8. Induction of chromosome aberrations

Cultures were set up by plating 5×10^5 cells into each 60-mm Petri dish and then incubating them overnight. Cells were treated with *N*-nitroso propoxur for 2 h, then incubated for 24 or 48 h. Two hours prior to the end of incubation, colcemid (0.2 μg/ml) was added to the culture. Mitotic cells were harvested by trypsinization, and slides were prepared by an air-dried technique. Slides were stained in a 3% Giemsa solution, coded, and blindly scored using an Olympus New Vanox photomicroscope. At least 100 metaphases were randomly sampled for the assay of chromosome aberrations.

2.9. HPRT gene mutations

SC-M1 cells (at 10^5) were grown in complete medium containing 100 μM hypoxanthine, 2 μM aminopterin, and 30 μM thymine (HAT medium, Sigma) for 3 days to reduce the number of preexisting HPRT-deficient cells. Cells (at 10^6/100-mm Petri dish) were plated and incubated overnight. Cultures were exposed to 0, 2, and 4 μg/ml *N*-nitroso propoxur for 2 h. At the end of treatment, the cultures were washed twice with PBS and re-fed with fresh medium. Cells were maintained in their proliferative growing state for an 8-day expression period before mutant selection. Afterward, 2×10^6 cells were divided among twenty 100-mm Petri dishes, fed complete media containing 10 μg/ml 6-thioguanine (6TG, Sigma), and incubated for 7 days. Plating efficiency was also determined at the same time by plating 100 cells in each 60-mm Petri dish with complete medium. The mutation frequency was determined as the number of 6TG-resistant colonies induced per 106 surviving cells.

2.10. Soft-agar colonization

The soft agar assay followed procedures described previously by other authors (Koi et al., 1989). Cells (10^4–10^5) with or without *N*-nitroso propoxur treatment were suspended in 0.3% Difco bactoagar in normal growth media containing 10% FBS and 0.1% bactopeptone. All

experiments were performed three times. The suspended cells were plated in 0.3% agar in 60-mm dishes above a layer of 0.6% agar containing normal media with 10% FBS and 0.1% bactopeptone. Dishes were incubated at 37 °C in 5% CO_2 for 10–14 days and then scored for colony growth.

2.11. Cell cycle analysis

SC-M1 cells (at 3×10^5) were plated in each 60-mm dish overnight before being treated with *N*-nitroso propoxur (2 μg/ml) for 2 h. Treated cells were then washed with 10 ml PBS twice and cultured for a series of different durations after treatment removal. Upon the end of each post-treatment culture, cells were trypsinized, fixed in 70% ethyl alcohol, and centrifuged at 1000 rpm for 5 min. The cell pellets were stained in a PI mixture containing 550 μl CMF-PBS, 200 μl of 5% Triton X-100, 200 μl of 20 μg/ml PI, and 50 μl of 2 μg/ml RNase A for 15 min in the dark at room temperature. Stained cells were then passed through a 40-μm nylon mesh and analyzed for cell cycle progression using a Coulter Epics XL-MCL flow cytometer with excitation at 488 nm and emission at 610 nm.

3. Results

3.1. Cell doubling time

The proliferation index of SC-M1 cells, which signifies the rounds of cell duplication completed within an indicated duration, was calculated by plating individual cells for 24, 44, 48, and 72 h as previously described above (Fig. 1). A regression equation was calculated from the logarithmically growing SC-M1 cells as: $f(x) = 0.0425\,x + 0.9781$, where $f(x)$ corresponds to the proliferation index after the duration, x(h). According to this equation, SC-M1 cells in the logarithmic phase doubled their population about every 24 h.

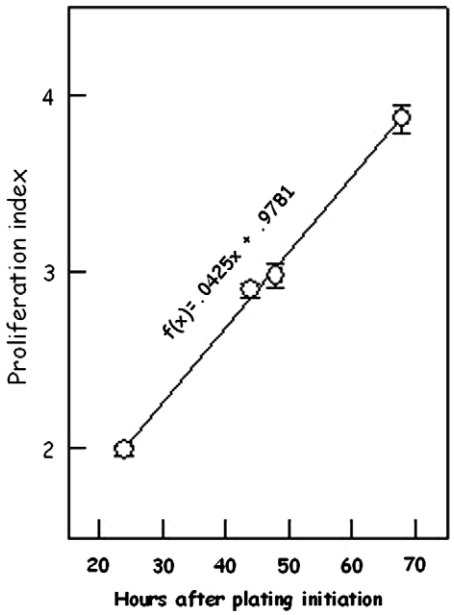

Fig. 1. Cytokinetics of human gastric SC-M1 cells. One thousand cells were plated into each grid 60-mm dish. Numbers of individual cell in each colony were counted 24, 44, 48 and 68 h after the initial plating using a dissecting microscope. Based on the distribution of the colony sizes, a proliferation index (PI) was derived according to the formula described in the text.

3.2. Growth inhibition

The dose–growth inhibition curve of *N*-nitroso propoxur-treated SC-M1 cells is shown in Fig. 2. A 2-h pulse treatment at doses of ⩽4 μg/ml showed growth inhibition of <10%. As the dose increased to 8 μg/ml, inhibition increased to about 20%. When treatments with *N*-nitroso propoxur were raised to 16 and 32 μg/ml, there was still about 50% growth observed. A J-shaped regression model better fit the dose–response curve than a linear one, with an *r*-value of 0.97 vs. 0.85, respectively. Determining whether this implies a hormetic-like relationship [Calabrese, 2003 #5] in which the cell growth rate exceeds the control value when the *N*-nitroso propoxur treatment dose becomes more dilute is worthy of further investigation.

3.3. Apoptosis

Whether the growth inhibition caused by *N*-nitroso propoxur treatment in SC-M1 cells was due to the induction of apoptosis was investigated using staining with Annexin-V in conjunction with PI (Fig. 3). It has been demonstrated that Annexin-V can bind to phosphatidylserine, which makes it a very useful tool for detection of apoptosis in the early stages of the process (Darzynkiewicz et al., 1997; van England et al., 1998). The frequency of SC-M1 cells positively stained for Annexin-V only, an indication of apoptosis, was less than 1% in both the control and *N*-nitroso propoxur-treated group up to 8 μg/ml. The positive control assay performed twice with 0.5 μM actinomycin D showed approximate 21% apoptotic cells. A 24-h

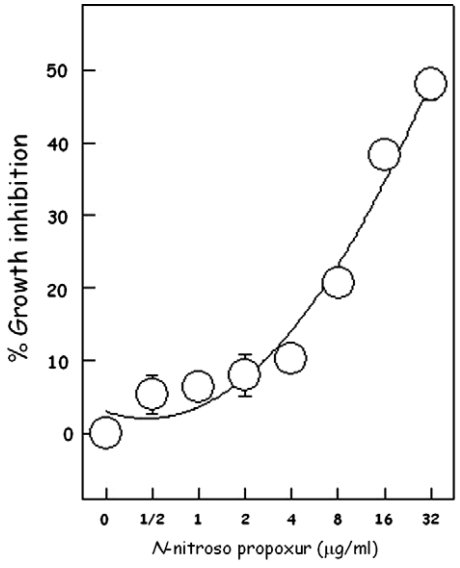

Fig. 2. Growth inhibition of *N*-nitroso propoxur to SC-M1 cells. Number of cells survived after treatment was approximated using UV absorption at 260 nm. Percent survival was calculated by dividing the absorbance of the treatment with that of the concurrent control.

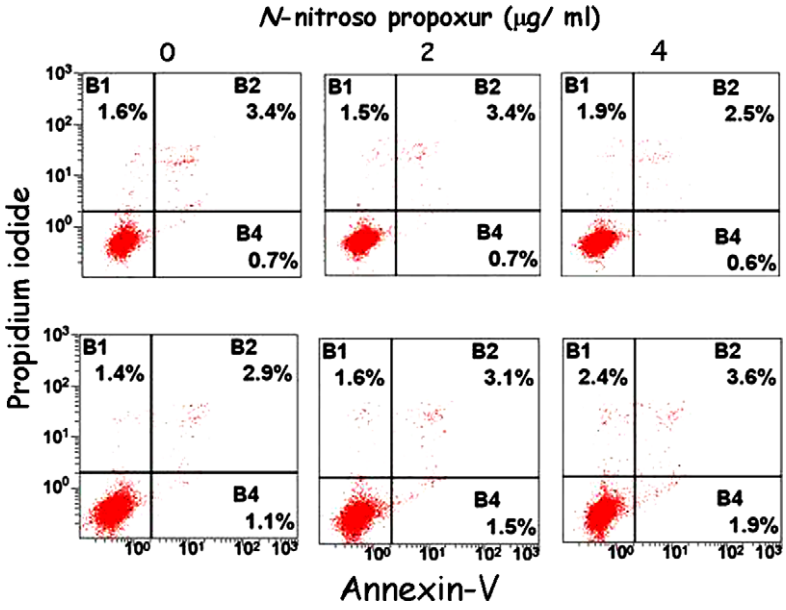

Fig. 3. Flow cytometric analysis of cell death induced by *N*-nitroso propoxur in SC-M1 cells. Cells were stained for propium iodine and Annexin-V after a 2 h pulsed-treatment with *N*-nitroso propoxur. Upper row indicates the results of assay immediately after the treatment, while the bottom row is those after a 24-h post-treatment incubation. Data shown in the figure were one example among three different experiments.

post-treatment incubation increased the frequency of Annexin-V staining, however, not significantly. The overall cell mortality, including that caused by apoptosis and necrosis, was less than 2.5% with treatment using *N*-nitroso propoxur at up to 4 μg/ml. This is consistent with the results of the growth inhibition assay described previously above. The portion of apoptotic cells which was positively stained by Annexin-V and negatively with PI never exceeded 4% of the total population in the treatment with *N*-nitroso propoxur at $\leqslant$ 4 μg/ml. Therefore, the growth inhibition caused by *N*-nitroso propoxur in our investigation was unlikely due to the induction of apoptosis.

3.4. DNA damage

N-nitroso propoxur-induced DNA damage was estimated using assays for single-cell electrophoresis (COMET) and the expression of phosphorylated histone H2AX (Banath and Olive, 2003). The DNA migration caused by *N*-nitroso propoxur treatment was measured by the tail moment in the COMET assay (Fig. 4). In 2 independent studies, treatments with 2 and 4 μg/ml *N*-nitroso propoxur greatly enhanced DNA migration. When an assay was performed immediately after treatment removal, the tail moments induced were around 66–95 times as high as the control treatment, respectively. The assay performed 24 h later, on the other hand, showed a great reduction in the tail moment in the treatment groups, implying that significant DNA repair had occurred during this period.

DNA damage was also analyzed using a flow cytometric method. SC-M1 cells were stained for DNA and γH2AX after *N*-nitroso propoxur treatment. A gate was drawn to exclude >95% of the cells of the untreated population,

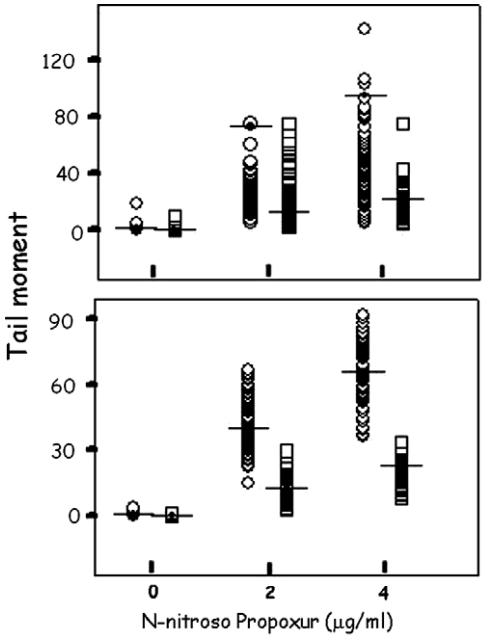

Fig. 4. COMET analysis of DNA damage induced by *N*-nitroso propoxur. Data shown are results of two independent experiments on COMET assay performed either immediately after a 2 h-treatment (open circle) or after a 24-h post-treatment incubation (open rectangular). Every individual comet recorded are shown in the figure. The horizontal bar indicates the medium in per 100 observations of each treatment.

and the fraction of cells within the gate indicated the specific level of γH2AX expression for each treatment. A significant dose–response induction of DNA strand breaks, mainly occurring in the S phase, was observed immediately after *N*-nitroso propoxur treatment (Fig. 5). The incidence of DNA damage investigated by this method also showed a significant reduction after the 24-h post-treatment incuba-

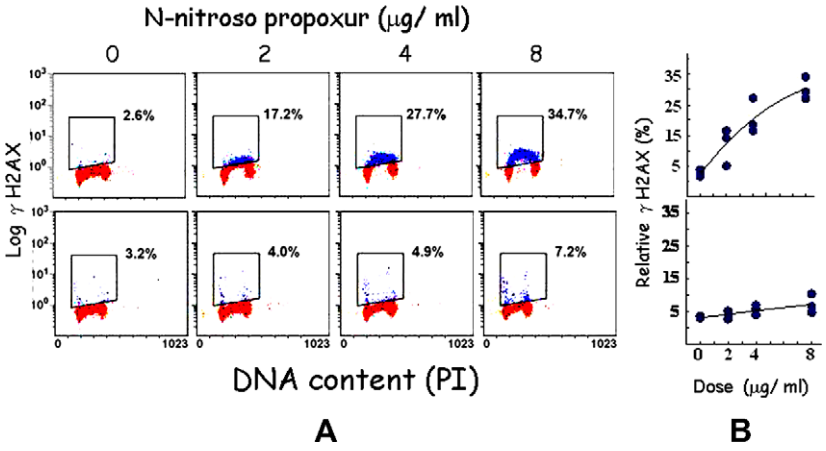

Fig. 5. Flow cytometric analysis of DNA damage induced by *N*-nitroso propoxur. SC-M1 cells stained for propidium iodine and γH2AX after a 2h-treatment with *N*-nitroso propoxur were shown in (A). An overall results of three different experiments were shown in (B). Upper row indicates the results of assay immediately after the treatment, while the bottom row is those after a 24-h post-treatment incubation.

tion, which is similar to the results found with the COMET assay. Therefore, *N*-nitroso propoxur treatment at 2 and 4 μg/ml was capable of damaging the DNA of SC-M1 cells. Most of the DNA damage, however, could be repaired within 24 h, i.e. within one replication cycle of SC-M1 cells.

3.5. Chromosome aberrations

A 2-h pulse treatment of *N*-nitroso propoxur with doses ⩽4 μg/ml was unable to induce significant chromosome aberrations in SC-M1 cells, although a previous investigation on COMET and γH2AX expression showed it caused significant DNA damage at those dose levels. *N*-nitroso propoxur is recognized as one of the potential compounds which produce O6-methylguanine adducts in mammalian cells in culture conditions (Wang et al., 1998b). These compounds were reported to yield chromosome aberrations with a delayed expression (Bean and Galloway, 1993; Galloway, 1994; Kaina et al., 1997). Therefore, we investigated the chromosome aberrations in cells harvested both 24 and 48 h after treatment removal, which are approximately equal to completion of 1 and 2 cell cycles in SC-M1 cells, respectively. However, neither of them showed significant induction of chromosome aberration (Table 1). Only when the treatment was increased up to 8 μg/ml did the induction of chromosome aberrations become significant. The delayed expression of chromosome aberrations was not found until the dose reached 8 μg/ml, with those incubated for 48 h showing higher chromosome aberrations than those incubated for 24 h. In treatment with 16 μg/ml *N*-nitroso propoxur, no mitotic cells were available for analysis in those cells harvested 24 h after the end of treatment, while significant chromosome aberrations were observed in those incubated for 48 h after treatment removal. The major type of chromosome aberration induced by *N*-nitroso propoxur was chromatid exchanges.

Table 1
Induction of chromosome aberrations in SC-M1 cells by *N*-nitroso propoxur

N-Nitroso propoxur (μg/ml)	Percent (%) aberrant cells[a]	
	Post-treatment incubation for	
	24 h	48 h
0	0	0
2	1	1
4	1	4
8	9**	15**
16	–[b]	53**

** Indicates a significant difference at the $p < 0.01$ level according to a binomial distribution.
[a] Cells with only a gap were not included.
[b] Analysis failed due to a lack of mitotic cells.

3.6. Hprt mutation

Induction of *hprt* gene mutation was expressed as the number of cells resistant to 6-thioguanine per 10^6 cells (Table 2). In two independent experiments, the frequency of 6-thioguanine resistance was $<10^{-6}$ in control SC-M1 cells. A 2-h treatment with 2 μg/ml *N*-nitroso propoxur increased the resistance frequency 10–20 times. The mutation frequency of the *hprt* gene in treatments with 4 μg/ml *N*-nitroso propoxur was 40–60 times as high as that of the control group.

Table 2
N-nitroso propoxur-induced 6-thioguanine (6-TG) resistance in SC-M1 cells

Dose (μg/ml)	Plating efficiency (%)	Number of 6-TG-resistant cells	
		2×10^6 cells	Per 10^6 surviving cells
Experiment 1			
0	102.3	2	0.97
2	99.3	21	10.6
4	100	121	60.5
Experiment 2			
0	80.7	1	0.6
2	148.3	71	23.9
4	91	71	39.1

3.7. Anchorage independence

Results of three independent studies showed that SC-M1 cells in the control and *N*-nitroso propoxur-treated (2 μg/ml, 2 h) groups did not significantly differ in plating efficiencies on plastic (Table 3). Their ability of soft-agar colonization, on the other hand, almost doubled after *N*-nitroso propoxur treatment. This indicated that the anchorage dependence in SC-M1 cells was significantly decreased by *N*-nitroso propoxur treatment at 2 μg/ml, which is a dose without apparent cytotoxic effects ($p < 0.01$, according to Student's *t*-test).

3.8. Perturbation of cell cycle progression

After 2 h of *N*-nitroso propoxur treatment (2 μg/ml), the logarithmically growing SC-M1 cells were incubated in *N*-nitroso propoxur-free normal media for a series of different time periods ranging from 2 to 24 h. In the first 6 h of the post-treatment period in normal media, cells from the control and treated groups showed similar patterns of cell cycle progression, with about 65–68% of cells remaining in the G1 stage, and 13% and 20% remaining in the S and G2/M stages, respectively (Fig. 6). Starting from around 8 h after *N*-nitroso propoxur removal, the effect of the treatment on cell cycle progression became apparent. The *N*-nitroso propoxur-treated cell population in the G1 stage gradually decreased, while that of the control group was still maintained at the previous level. The G1 population of cells collected during 12–16 h after *N*-nitroso propoxur removal was 20% less than that collected from the control group at the same period. After that, the G1 population in *N*-nitroso propoxur-treated cells gradually increased again and returned to the same level as the control group 24 h after *N*-nitroso propoxur removal. During the period when the G1 population in *N*-nitroso propoxur-treated cells decreased, i.e. between 8 and 16 h after *N*-nitroso propoxur removal, the G2/M population was found to have concomitantly increased. In treated cells collected 16 h after *N*-nitroso propoxur removal, 50% of them stayed in the G2/M phase. In the control treatment, only 20% of G2/M cells were collected in the same period. Therefore, *N*-nitroso propoxur treatment induced transient cell cycle arrest at the G2/M stage, and consecutively caused the cell population to decrease in the G1 stage. Deviations also found in S-phase cells between the control and *N*-nitroso propoxur treatments, however, were not as apparent.

Table 3
N-nitroso propoxur-induced anchorage independence in gastric SC-M1 cells

N-nitroso propoxur (μg/ml)	Plating efficiency		
	In soft agar	On plastic	Per 100 surviving cells in soft agar
0	14.8 ± 2.8	77.1 ± 12.3	19.1 ± 0.9
2	29.0 ± 4.0**	83.0 ± 12.7	36.2 ± 1.1**

** Indicates a significant difference from the control at $p < 0.01$, according to Student's *t*-test.

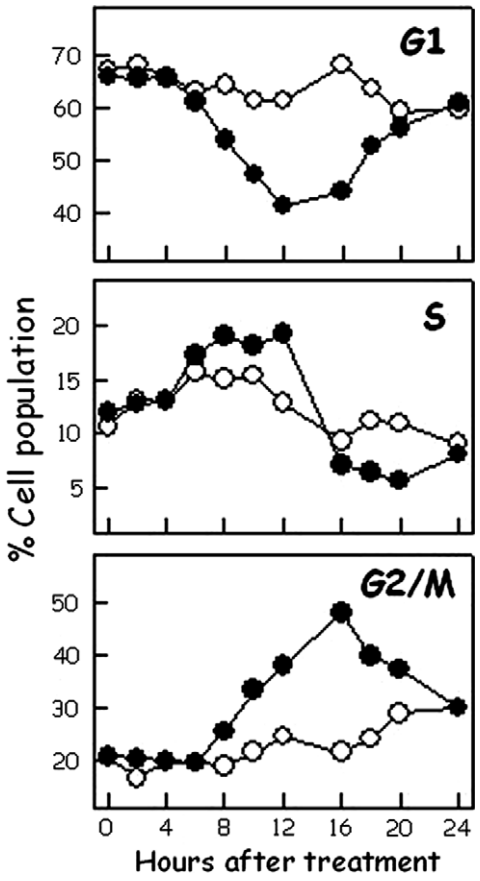

Fig. 6. Perturbation of cell cycle progression by *N*-nitroso propoxur. SC-M1 cells were treated with control media (open circle) or 2 μg/ml *N*-nitroso propoxur for 2 h. The cells were then harvested for cell cycle analysis after incubated in normal media for a series of different period.

4. Discussion

N-Methylcarbamate insecticides hold a special position among many pesticides used in pest control programs. They serve as possible precursors of *N*-nitroso compounds, and thus they probably play key roles in the development of gastric cancer in humans (Palli et al., 2001). The suggestion that the formation of mutagenic metabolites should be included as a parameter in the toxicological evaluation of carbamate insecticides was proposed as early as the 1970s (Uchiyama et al., 1975) when the interaction of the *N*-methylcarbamate insecticide, carbaryl, with sodium nitrite was demonstrated to produce *N*-nitroso carbaryl (Elespuru and Lijinsky, 1973; Siebert and Eisenbrand, 1974). In the following years, numbers of *N*-methylcarbamate insecticides that were shown to convert into *N*-nitroso metabolites have accumulated. Among *N*-methylcarbamate insecticides, propoxur is one of the most extensively used pest insect control agents in Taiwan. The possibility of it contaminating food, water, and air, thereby producing adverse effects in humans, is inevitable. *In vitro* studies on Chinese hamster V79 and primary rat tracheal epithelial cells demonstrated solid evidence that the *N*-nitroso derivative of propoxur is highly genotoxic to mammalian cells

(Wang et al., 1998a,b). What we present in this report are results of an investigations carried out on human gastric cells. This should be more relevant to the elucidation of its adverse effect since the stomach has been recognized as the major target for the *N*-nitroso *N*-methylcarbamate insecticides (Lijinsky, 1992).

In addition to using target cells of *N*-nitroso *N*-methylcarbamate to obtain pertinent results, this report revealed the potential risks of low doses of *N*-nitroso propoxur to human health which have not been disclosed in previous investigations. Compared with Chinese hamster V79 and primary rat tracheal epithelial cells, human gastric SC-M1 cells are much more resistant to *N*-nitroso propoxur. A 2-h pulse treatment of *N*-nitroso propoxur at 2 μg/ml induced a mortality of $\geqslant 50\%$ in V79 and primary rat tracheal epithelial cells (Wang et al., 1998b). In SC-M1 cells, on the other hand, more than 90% of cells survived with the same treatment. When the treatment dose of *N*-nitroso propoxur increased to 4 μg/ml, 90% of SC-M1 cells continued to grow, while 90% of V79 cells were killed. At these non-cytotoxic dose levels, i.e. doses $\leqslant 4$ μg/ml, *N*-nitroso propoxur nevertheless induced a considerable amount of DNA damage in SC-M1 cells, which was identified by both COMET and γH2AX staining assays. The DNA damage in SC-M1 cells induced by *N*-nitroso propoxur treatment at those dose levels, however, did not seem likely to turn into aberrant chromosomes. The cytogenetic assay indicated that at these dose levels, both the regular and delayed harvest protocols failed to detect significant chromosome aberrations. The phenomenon that *N*-nitroso propoxur induced DNA damage but not chromosome aberrations is probably due to most of the DNA damage produced by low-dose *N*-nitroso propoxur treatment being repaired before cells entered the mitotic stage. This is true because both the COMET and γH2AX staining assays showed that DNA damage significantly decreased after a 24-h post-treatment incubation in drug-free media. Cell cycle analysis further revealed that a transient G2/M arrest was observed in *N*-nitroso propoxur-treated cells starting from the 8th hour after treatment removal. The transient G2/M arrest provides a protective mechanism to ensure repair of induced DNA damage and prevented inappropriate mitotic entry so that we were unable to find significant induction of chromosome aberrations in low-dose treatments of *N*-nitroso propoxur.

Although *N*-nitroso propoxur treatments at non-cytotoxic dose levels did not induce significant chromosome aberrations, they did provoke significant gene mutations at the *hprt* locus in SC-M1 cells. The induction of *hprt* mutations has been interpreted as one of the biomarkers revealing an increase in genetic instability (Albertini et al., 1998; Finette et al., 2000; Kendall et al., 2004) and as being a mutational process with carcinogenic potential (Albertini, 2001). The significant induction of *hprt* gene mutations in this report implies that while the chromosome integrity in *N*-nitroso propoxur-treated SC-M1 cells was maintained through DNA repair during the transient G2/M arrest period, their concurrent genetic stability did not seem to be properly preserved. Therefore, exposure to low doses of *N*-nitroso propoxur has the potential to accelerate spontaneous genetic changes in human gastric cells without showing an apparent growth inhibition effect or gross chromosome alterations. The increase in genetic instability has the potential to progress to cellular malignancy. Our results in the soft-agar assay provide auxiliary evidence for elucidation of this phenomenon. Soft-agar colonization, an indication of anchorage-dependent loss, is commonly recognized as one of many phenomena characterizing the progression to cellular malignancy (Allan et al., 2006; Gao et al., 2005). After being treated with 2 μg/ml *N*-nitroso propoxur, twice as many SC-M1 cells grew in soft-agar colonization as in the controls. This implies that low-dose *N*-nitroso propoxur exposure not only increased the genetic instability but also pushed the development of malignancy forward in SC-M1 cells. Contamination by propoxur in the environment, due to its potential to convert to *N*-nitroso derivatives, is thus a great concern to human health.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

This research was supported by Grants from the Institute of Cellular and Organismic Biology, Academia Sinica in Taiwan.

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