

DNA Binding and stimulation of cell division in the carcinogenicity of styrene 7,8-oxide

W.K. Lutz, S. Cantoreggi and I. Velic

*Institute of Toxicology, Swiss Federal Institute of Technology
and University of Zürich, Schwerzenbach, Switzerland*

Summary. [7-³H]Styrene 7,8-oxide was administered by oral gavage to male CD rats at a dose of 1.3 mg/kg. After 4 h, the forestomach was excised, DNA was isolated, purified to constant specific radioactivity and degraded enzymatically to the 3'-nucleotides. High-performance liquid chromatography fractions with the normal nucleotides contained most of the radiolabel, but a minute level of adduct label was also detected. Using the units of the covalent binding index (micromoles adduct per mole DNA nucleotide)/(millimole chemical administered per kilogram body weight), a DNA binding potency of 1.0 was derived. A comparison of the covalent binding indices and carcinogenic potencies of other genotoxic forestomach carcinogens showed that the tumorigenic activity of styrene oxide is unlikely to be purely genotoxic. Therefore, styrene oxide was compared with 3-*t*-butylhydroxyanisole (BHA) with respect to stimulation of cell proliferation in the forestomach. Male Fischer 344 rats were treated for four weeks at three dose levels of styrene oxide (0, 137, 275 and 550 mg/kg, three times per week by oral gavage) and BHA (0, 0.5, 1 and 2% in the diet); the highest doses had been reported to result in 84% and 22% carcinomas in the forestomach, respectively. Cell proliferation was assessed by incorporation of bromodeoxyuridine into DNA and immunohistochemical analysis. An increase in the labelling index was found in all treated animals. In the prefundic region of the forestomach, the labelling index increased significantly, from 42% (controls) to 54% with styrene oxide and from 41 to 55% with BHA. Rats treated with BHA also had severe hyperplastic lesions in the prefundic region, i.e., at the location of BHA-induced forestomach carcinomas. The number of cells per millimetre of section length was increased up to 19 fold. Hyperplastic lesions were not seen with styrene oxide, despite the higher tumour incidence reported with this compound. We conclude that the carcinogenicity of styrene oxide to the forestomach most probably involves a mechanism in which marginal genotoxicity is combined with promotion by increased cell proliferation.

Introduction

Carcinogenesis is a multi-stage process which results in an accumulation of a number of critical changes in DNA (Harris, 1991). The rate of this process is dependent on the level of primary DNA lesions and on the rate of DNA replication, which can result in fixation of the lesions as heritable mutations. Whether a chemical carcinogen affects the first or the second aspect is important because the extrapolation to low doses would probably follow different rules (Lutz, 1990). While DNA adduct formation is expected to be proportional to dose at the lowest levels, cell division might not be affected below a certain dose. This question was addressed in interpreting the results of bioassays with styrene 7,8-oxide.

Styrene oxide has been investigated for carcinogenicity in several studies in animals: After oral gavage, forestomach tumours were induced in both rats and mice (IARC, 1985; Lijinsky, 1986). The genetic toxicology of styrene and styrene oxide has been reviewed recently (Barale, 1991). Styrene oxide was weakly mutagenic in a number of test systems without metabolic activation. DNA adduct formation by styrene oxide *in vitro* has been investigated by Hemminki and coworkers (Vodicka & Hemminki, 1988, and references therein). One publication reported the putative identification of a 7-guanyl adduct in liver DNA isolated from mice after intraperitoneal administration of tritiated styrene and styrene oxide (Byfält-Nordqvist *et al.*, 1985). The DNA binding potency tentatively estimated from that study and expressed in the units of the covalent binding index (CBI) for liver DNA (Lutz, 1979)—(micromoles adduct per mole DNA nucleotide)/(millimole chemical administered per kilogram body weight)—was around 2 for styrene oxide and 2–5 for styrene. This potency was higher than expected from the data on genotoxicity *in vitro* and from the low chemical reactivity of styrene oxide (Betso *et al.*, 1991). Reinvestigation of the DNA binding potency of styrene oxide in this laboratory showed no detectable DNA adduct formation, with a limit of detection of CBI < 2.6 for forestomach DNA in the rat (Cantoreggi & Lutz, 1992).

This study was designed to answer the following questions: (i) whether DNA adduct formation by styrene oxide in the forestomach could be detected if a lower limit of detection were achieved, and (ii) whether stimulation of cell division is the mechanistic basis for induction of forestomach tumours by styrene oxide.

Materials and methods

Styrene oxide–DNA binding

Chemicals, animals, treatment

[7-³H]Styrene oxide (103 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, United Kingdom. Male Crl:CD[®]BR rats from Charles River Wiga, Sulzfeld, Germany, were used. Radiolabelled styrene oxide was dissolved in dimethyl sulfoxide and corn oil, as described previously (Cantoreggi & Lutz, 1992) and administered to six rats. The exact dose was determined on the basis of the weight of the solution administered and is given in Table 1.

Isolation of DNA and adduct analysis

After 4 h, forestomachs were excised, pooled into two groups and kept frozen at –20 °C. DNA was isolated by chromatin (Sagelsdorff *et al.*, 1983). Repurification to constant specific activity was carried out as reported earlier (Cantoreggi & Lutz, 1992). DNA was digested to

constituent deoxyribonucleotides (Sagelsdorff *et al.*, 1988), which were separated by high-performance liquid chromatography on an Eurosphere RP18 5 μ m column (250 \times 4 mm) with 2 ml/min 30 mM ammonium formate buffer, pH 3.8 and a 2–60% methanol gradient system (Cantoreggi & Lutz, 1992). The radiolabel attributable to adducts was converted to CBI units.

Cell division in the forestomach after exposures to styrene oxide and BHA

Application solutions and food

Solutions of styrene oxide (purity, > 98%; Merck) were prepared in corn oil (Kentaur-Nuxo, Burgdorf, Switzerland). 3-*t*-Butyl-4-hydroxyanisole (BHA; purity, > 98%) was obtained from Fluka (Buchs SG, Switzerland) and cereal-based maintenance diet (#890) in pellets and powder form from Nafag (Gossau SG, Switzerland). The appropriate amounts of BHA (e.g., 60 g for the 2% diet) were dissolved in 100 ml diethyl ether and poured onto 400 g powdered food. A homogeneous slurry was prepared, and the ether was evaporated under ambient air for 60 h. The premix was pulverized in a mortar and mixed for 0.5 h in a food-mixing machine to make up a total of 3 kg.

Animals and treatments

Forty seven-week-old male Fischer 344 rats from Charles River Wiga were held in groups of five. Those exposed to styrene oxide received pelleted diet at all times, whereas those treated with BHA were switched to powdered food after one week of acclimatization. During the four-week treatment period, rats were administered styrene oxide at 0, 137, 275 or 550 mg/kg by oral gavage in corn oil (1 ml solution per kg bw), three times per week (on Monday, Wednesday and Friday at 8:00 h). The concentrations of BHA in powdered food were 0, 0.5, 1 and 2%.

On Monday of week 5, after a final treatment with styrene oxide, Alzet osmotic minipumps (Model 2001D, Lot 047001, flow rate 9.1 μ l/h, one-day delivery; Alza Corp., Palo Alto, CA, USA) filled with 220 μ l bromodeoxyuridine (BrdU) (Sigma) solution (20 mg/ml BrdU, 10 mM potassium phosphate, 130 mM sodium chloride, pH 7.6, and 1% 1 N sodium hydroxide) were implanted subcutaneously in the upper back of the rats in the early afternoon.

Histopathology and immunohistochemistry

After 24 h, the stomach was excised, cut open along the minor curvature, rinsed in 0.9% aqueous sodium chloride, pinned flat on a cork board and fixed in buffered formalin for one week. A 3-mm thick strip was cut along the major curvature, embedded in Paraplast paraffin (Monoject Scientific Inc., Athy, Ireland) and cut into 2- μ m-thin sections.

The sections were stained immunohistochemically for incorporation of BrdU into DNA (Dietrich & Swenberg, 1991). A monoclonal primary antibody (anti-BrdU murine immunoglobulin G; Becton Dickinson Co., Research Triangle Park, NC, USA) was linked with a secondary antibody (biotinylated goat anti-mouse immunoglobulin G) to a label (streptavidin) coupled with alkaline phosphatase. Fast red was used as the chromogen. Link and label were components of a 'Supersensitive Kit' purchased from BioGenex Laboratories Inc. (San Ramon, CA, USA). The sections were counterstained with haematoxylin.

Assessment of cell numbers and labelling index

BrdU-positive cells had red nuclei; the negative nuclei stained blue. Cells (220 on average) were counted in three defined anatomical regions of the forestomach: the saccus

caecus (near the oesophagus), the midregion and the prefundic region (near the limiting ridge with the glandular stomach). Only the cells in the basal, cohesive layers were counted. The total number of cells per millimetre section length (1), the number of dividing cells per millimetre section length (2) and the labelling index, the ratio of (2):(1), were determined. One section was evaluated per rat. An average was calculated for the groups of five rats and expressed as mean \pm 1 SD. Differences between the groups were analysed using a two-sided Fisher test.

Results

Styrene oxide–DNA binding

Forestomach DNA samples isolated from treated animals showed a constant specific radioactivity after one round of repurification (Table 1). Radiolabel irreversibly associated with DNA is not necessarily due to nucleotide–carcinogen adduct formation but can be the result of biosynthetic incorporation of radiolabel during DNA biosynthesis. With [7-³H]styrene oxide, for instance, tritiated water is formed during metabolism. Tritium from water can be incorporated into the 2'-position of the deoxyribose in the ribonucleotide reductase step. In order to distinguish true adduct formation from biosynthetic radiolabel incorporation, nucleotides were analysed for radiolabel.

Table 1. Covalent binding of [7-³H]styrene 7,8-oxide (SO) to forestomach DNA of male CD rats after oral administration

Rat no.	Dose		DNA specific activity (dpm/mg)*		Nucleotide analysis			CBI units
	mg/kg	10 ⁹ dpm/kg	1st purification	2nd purification	Radiolabel recovered	Radiolabel without OD	SO–nucleotide adducts	
1	1.41	2.67						
2	1.37	2.60	51 \pm 8	45 \pm 9				
3	1.25	2.39			60%	39%	8.2 dpm/mg	1.0
4	1.28	2.44						
5	1.24	2.38	33 \pm 8	25 \pm 8				
6	1.25	2.39						

OD, optical density; \pm 2 SD from the statistical counting error

High-performance liquid chromatography profiles of nucleotides showed that most of the radiolabel eluted with the normal nucleotides. A small but significant amount of radiolabel was detected at elution times similar to those of the early-eluting styrene–nucleotide adducts (Cantoreggi & Lutz, 1992). When expressed as CBI, the DNA binding potency was at the low level of 1.0.

Effects of styrene oxide and BHA on the forestomach

Histopathology

In the styrene oxide-treated rats, a marginal increase in the thickness of the squamous epithelium and slightly enhanced keratinization were observed. Two animals, one in the intermediate- and one in the high-dose group, showed mild hyperplasia in the midregion.

BHA treatment resulted in a dose-dependent increase in the number and severity of histopathological lesions in the forestomach. At a dose of 0.5% BHA, there was mild hyperkeratinization and increased thickness of the squamous epithelial layer. One animal had a mildly hyperplastic lesion near the limiting ridge. All animals treated with 1% BHA showed squamous epithelial hyperplasia and hyperkeratosis near the limiting ridge, while few animals had hyperplastic lesions in the midregion of the forestomach. No lesion was observed in the saccus caecus. Various degrees of hyperplasia were seen in all rats in the group treated with 2% BHA; in one case, possibly a papilloma was seen near the limiting ridge. In three animals, mild hyperplastic lesions were found in the midregion of the forestomach. Hyperkeratinization was evident in all three anatomical regions of the forestomach, although it was most extensive near the limiting ridge and in the midregion.

Labelling index

The four-week exposure of rats to both styrene oxide and BHA significantly increased the rate of proliferation in all regions of the forestomach (Table 2). No clear dose-response relationship was seen, indicating that the cell proliferation response had reached its biological maximum when measured as an integral over 24 h. Styrene oxide and BHA had similar effects. The markedly different pathological findings were not reflected by the labelling index.

Table 2. Labelling index (BrdU-positive cells divided by total cells x 100) in the basal-cell layers in three regions of the forestomach of male Fischer 344 rats exposed by oral gavage to styrene 7,8-oxide (three times per week for four weeks) and 3-t-butyl-4-hydroxyanisole (BHA) in the diet for four weeks (mean \pm 1 SD)

Forestomach region	Styrene oxide dose (mg/kg)				BHA concentration in diet (%)			
	0	137	275	550	0	0.5	1	2
Saccus caecus	49 \pm 8	61 \pm 6**	70 \pm 5***	70 \pm 4***	37 \pm 7	50 \pm 10**	53 \pm 10**	51 \pm 9*
Midregion	43 \pm 1	63 \pm 8***	63 \pm 7***	59 \pm 5***	43 \pm 7	66 \pm 4***	56 \pm 14*	60 \pm 15**
Prefundic region	42 \pm 8	47 \pm 9	55 \pm 9*	54 \pm 11*	41 \pm 6	62 \pm 8***	66 \pm 8***	55 \pm 8**

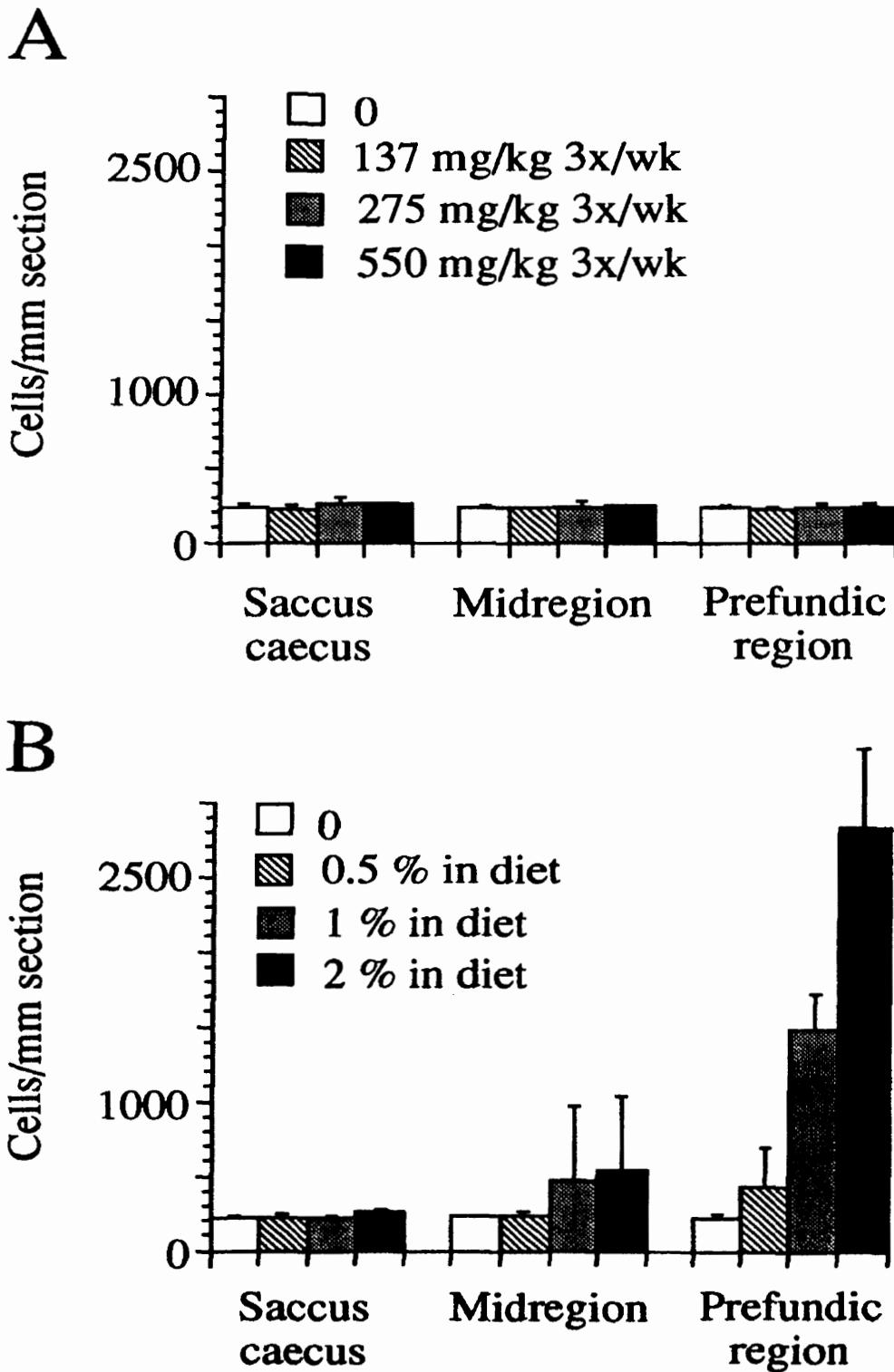
Differences from control group (two-sided Fisher test): *, $p < 0.05$; **, $p < 0.025$; ***, $p < 0.0025$

Quantification of hyperplasia

As the labelling index did not indicate regional differences, yet pathological observations correlated with tumour location after BHA treatment, additional variables were investigated for a quantitative description of the hyperplastic response of the forestomach to BHA. The total number of cells per millimetre section length is shown in Figure 1. In rats given styrene oxide, this number was constant, in agreement with the lack of site specificity of the (minor) histopathological findings. Dietary exposure to BHA, however, caused a dramatic increase, mostly in the prefundic region, in the number of cells per unit section length. At the lowest dose (0.5%), two of five animals showed a three-fold increase in the number of cells per millimetre section length in the prefundic region of the forestomach. No hyperplastic effect was seen in the saccus caecus or in the midregion. With 1% BHA, all animals showed a marked increase in the number of cells in the prefundic region; in the group given 2%, the

effect was even more dramatic, and one animal had a 19-fold increase in the number of cells per millimetre over that in controls. The saccus caecus region was still not affected.

Figure 1. Number of cells per millimetre section length in three regions of the forestomach of male Fischer 344 rats exposed to styrene oxide (A) by oral gavage (three times per week for four weeks) and 3-*t*-butyl-4-hydroxyanisole (BHA; B) at 0–2% in the diet for four weeks. Means \pm 1 SD



Discussion

DNA binding potency of styrene oxide and genotoxic carcinogenesis

Styrene oxide was shown to have low DNA binding potency in the forestomach of rats, with a CBI of 1.0. This low genotoxic potency is in concordance with the weak mutagenicity of styrene oxide (Barale, 1991) and with its poor reactivity towards DNA *in vitro* (Cantoreggi & Lutz, 1992).

Styrene had a CBI of only 0.1 in mouse liver and rat lung after exposure by inhalation (Cantoreggi & Lutz, 1993), although styrene is metabolized almost completely to styrene oxide. The 10-fold greater DNA binding potency of styrene oxide in the forestomach is the result of a high local concentration of the compound at the site of administration.

The aim of this study was to investigate whether DNA binding is responsible for the tumorigenic response observed. This goal can be accomplished by comparing the CBI of styrene oxide with those of other forestomach carcinogens. For instance, 1,2-dichloroethane produced forestomach tumours in rats after administration by oral gavage, with a carcinogenic potency similar to that of styrene oxide (Gold *et al.*, 1991). Reitz *et al.* (1982) showed that 1,2-dichloroethane has a CBI of 7 in the stomach, i.e., a DNA binding potency more than seven times that of styrene oxide. This finding may be a first indication that a purely genotoxic mechanism for the tumorigenic action of styrene oxide in the forestomach is unlikely.

Effects of styrene oxide and BHA on the forestomach

The two forestomach carcinogens, styrene oxide and BHA, showed different dose-response relationships for cancer induction. At the highest dose level used in both the bioassays and the present study, styrene oxide induced squamous-cell carcinomas in male rats at an incidence of 84% (Lijinsky, 1986); BHA induced an incidence of 22% (Ito *et al.*, 1986). At one-half of that dose, styrene oxide still induced forestomach carcinomas in 67% of the rats, whereas BHA induced benign lesions only. On the basis of this dose-effect relationship, we would have expected a generally stronger response in the styrene oxide-treated animals, but, with respect to hyperplasia and the number of cells 'at risk', the opposite was true: BHA was much more effective. Additional mechanisms of action must be operating in the induction of the high incidence of carcinomas by styrene oxide. In view of its mutagenic activity and of the DNA binding detected in this study, genotoxicity is a reasonable explanation. The high incidence of forestomach tumours is therefore probably due to a combination of DNA adduct formation and an enhanced rate of cell proliferation.

If this combination resulted in multiplicative synergism of genotoxic and epigenetic mechanisms, it would also affect the shape of the dose-response curve: The level of DNA damage would be expected to decrease in proportion to decreasing dose. For cell division, however, no increase would be expected at dose levels that no longer result in stimulation of cell division, resulting in non-linearity for forestomach tumours induced by low doses of styrene oxide. Under such circumstances, estimates of carcinogenic potency derived from exposures at high dose levels should not be used to predict the effects of low-level exposures.

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Note added in proof:

A full description of the study of cell proliferation has appeared in *Cancer Research*, **53**, 3505–3508 (1993): Induction of cell proliferation in the forestomach of F344 rats following subchronic administration of styrene 7,8-oxide and butylated hydroxyanisole, by S. Cantoreggi, D.R. Dietrich and W.K. Lutz.