Vol. 152, No. 1, 1988
April 15, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 423-431

CHRONIC ETHANOL CONSUMPTION INHIBITS REPAIR OF DIMETHYLNITROSAMINE-INDUCED DNA ALKYLATION

Siraj I. Mufti[*,1], Mario Salvagnini[2,+], Charles S. Lieber[+], and Anthony J. Garro[#]

*Department of Microbiology, The Mount Sinai School of Medicine, New York, NY 10029

+Veterans Administration Medical Center, The Alcohol Research and Treatment Center, Bronx, NY 10461

#The Sophie Davis School of Biomedical Education, The City of College of New York, New York, NY 10031

Received March 4, 1988

SUMMARY: Chronic ethanol consumption causes a DNA repair deficiency. This was demonstrated in Sprague-Dawley rats injected with ^{14}C-labeled dimethylnitrosamine after being pair-fed isocaloric, ethanol, or carbohydrate control diets for 4 weeks. Hepatic DNA was isolated from rats killed at intervals over a 36 hour period after administration of the nitrosamine and concentrations of alkylated guanine derivatives were measured. While N7-methylguanine was lost at equivalent rates from the DNA of both diet groups, O^{6}methylguanine, a promutagenic lesion, persisted at higher levels for longer periods of time in the DNA from the alcohol-fed animals.

Chronic alcohol consumption is associated with an increased risk of cancers in humans (for example, see 1-8). At present, it is not known how ethanol causes increased cancers, but it is generally assumed that ethanol per se is not a carcinogen and, therefore, ethanol effects need be explained in terms of its modifying the effects of other carcinogens. Ethanol is known to induce the enzymes of the microsomal P-450 cytochrome system (9) that metabolize and activate carcinogens into reactive intermediates (10,11). It has been assumed, therefore, that ethanol would increase the intermediates that react with DNA and thereby cause an increase in the putative mutagenic/carcinogenic lesions.

In order to determine if ethanol would cause an increase in mutagenic/carcinogenic lesions, we used dimethylnitrosamine (DMN), a potent hepatocarcinogen that alkylates DNA (12-14). The DNA base guanine is the

Present Addresses: [1]Department of Pharmacology and Toxicology, University of Arizona Health Sciences Center, Tucson, Arizona 85721; [2]Clinica Medica, Policlinico University, Padova, Italy.

0006-291X/88 $1.50

main target of alkylation and N7-methylguanine (7meG) is the major product of this alkylation induced by DMN (15,16). However while 7meG has normal base pairing properties and is not mutagenic, O^6methylguanine (O^6meG), a minor alkylated product formed at levels of 1 to 10% of 7meG, mispairs (17,18) and the formation and persistence of this base has been correlated with the mutagenic and carcinogenic properties of DMN (19-24). We wanted to determine the effect of ethanol on the formation and persistence of these DNA lesions resulting from treating experimental rats with DMN.

MATERIALS AND METHODS

Chemicals. Radioactively labeled di-^{14}C-methylnitrosamine (^{14}C-DMN) was purchased from Amersham Radiochemicals; it was diluted with unlabeled DMN purchased from Aldrich Chemicals. Small quantities of the radiochemical were obtained for treating animals and it was properly sealed, stored, and handled only when used; all instructions of the Radiation Control and Safety Division were adhered to for use and disposal of the radioactive material. Aquasol NEF934 used for radioactivity counting was purchased from New England Nuclear.

All other chemicals were purchased from Sigma Chemical Company.

Animals and their treatment. Male Sprague-Dawley rats weighing 125-150 gram were obtained from the animal facility maintained at the Veterans Administration Medical Center in Bronx, New York. The experimental rats were pair-fed nutritionally adequate, isocaloric liquid diet; part of carbohydrates of the control diet was substituted with ethanol for ethanol-consuming rats such that ethanol constituted 36% of the total caloric intake (25,26). The rats were fed control or isocaloric ethanol diet for four weeks. After an overnight fast, the animals were injected i.p. with a single dose of ^{14}C-DMN in 0.9% (w/v) NaCl at the rate of 1.5 mg (3.70 mCi/mmol)/kg, 10 mg (0.62 mCi/mmol)/kg and 25 mg (0.25 mCi/mmol)/kg body weight (using ^{14}C-DMN, 12 mCi/mmol which had been diluted with unlabeled DMN). The animals were killed at different time intervals over a 36 hr period and during this time they were provided with an adequate amount of carbohydrate control diet.

Isolation of hepatic DNA. Livers of treated animals were excised, homogenized, and their DNA isolated by phenol extraction according to the procedure of Margison and Kleihues (20). Contaminating RNAs were removed by digestion with a combination of pancreatic and T1 ribonucleases (0.2 mg/ml of each). Following this, DNA was precipitated with ethoxyethanol, air dried, and resuspended in 2 ml of water. The final concentration of the DNA preparations ranged from 2-3 mg/ml and RNA contamination was estimated at less than 0.2% of the total DNA as determined by residual counts of ^{32}P-labeled RNA added as a marker to sample liver homogenates.

Determination of methylated and unmethylated DNA bases. The concentrations of O^6meG and 7meG relative to guanine were determined as described by Montesano et al. (27). In essence, the DNA samples were hydrolyzed with 0.1 M HCl at 37°C for 16 h and the liberated purines were separated by chromatography on Sephadex G-10 columns. One hundred forty fractions of 5 ml each were collected and the concentrations of guanine and adenine in each sample were determined by absorbance at 160 nm. Ten ml of Aquasol NEF934 then was added to each sample and the concentrations of 7meG and O^6meG were determined by radioactivity counting in a Beckman LS9000 scintillation counter. Sufficient DNA was used to produce at least 100 cpm

above background in the O^6meG peak. The elution positions of the isolated bases from the Sephadex columns were verified by reference to known standards. The ratio of adenine to guanine in the hydrolyzates was 1.4 and corresponded to that expected for rat DNA.

RESULTS

Hepatic DNA was isolated from Sprague Dawley rats that were administered ethanol for four weeks, treated with a single dose of DMN at the rate of 1.5 mg, 10 mg and 25 mg/kg and killed at various intervals after DMN administration. The hepatic DNA was then subjected to chromatographic separation on Sephadex G-10 as described above. The data on the levels of methylated guanines 7meG and O^6meG, expressed as moles relative to guanine are presented in Fig. 1. Since 7meG is the major alkylation product of the reaction of DMN with DNA, the levels of 7meG correspond to total alkylation of DNA. The results (panels A, C and E) indicate that at the higher doses of 25 mg/kg and 10 mg/kg DNA was maximally alkylated by 12 hr after DMN administration, whereas at the 1.5 mg/kg dose maximum alkylation had occurred by 4 hr. However, the data do not indicate any consistent or significant differences in the initial or maximal 7meG levels in DNA obtained from ethanol consuming animals when compared to their controls. Following the maximal alkylation, a loss in 7meG levels is observed at subsequent time points: the loss of 7meG in the two diet groups also does not appear significantly different.

The levels of O^6meG as moles relative to guanine are shown in panels B, D and F of Fig. 1. Conforming to the data for 7meG, the higher doses of 25 mg/kg and 10 mg/kg produced maximum O^6meG levels at 12 hr while with the 1.5 mg/kg dose maximum O^6meG levels were observed by 4 hr. Here again, there were no consistent differences in the initial or maximum levels of O^6meG achieved in the DNA from ethanol-consuming versus control-diet rats. However, whereas subsequent to maximum alkylation there was no significant difference in the level of 7meG from the two diet groups as discussed above, consistently larger amounts of O^6meG persisted in DNA from the rats that consumed ethanol. With the 1.5 mg/kg DMN dose there was no detectable loss of O^6meG from DNA of the ethanol-diet animals over the 36 hr observation period, while about 80% of the initial O^6meG had been removed from DNA of the control rats by this time. At the 10 mg/kg and 25 mg/kg DMN doses, the levels of O^6meG persisting at 12 hr in DNA of ethanol-diet animals were about 1.5 to 2 times that of the controls and these differences were still evident at 36 hr. In contrast to O^6meG, 7meG was lost at approximately the same rate from DNA of both diet groups (panels A, C and E).

The greater relative persistence of O^6meG in DNA of the ethanol-fed animals is shown more clearly when plotted as the ratio of O^6meG relative

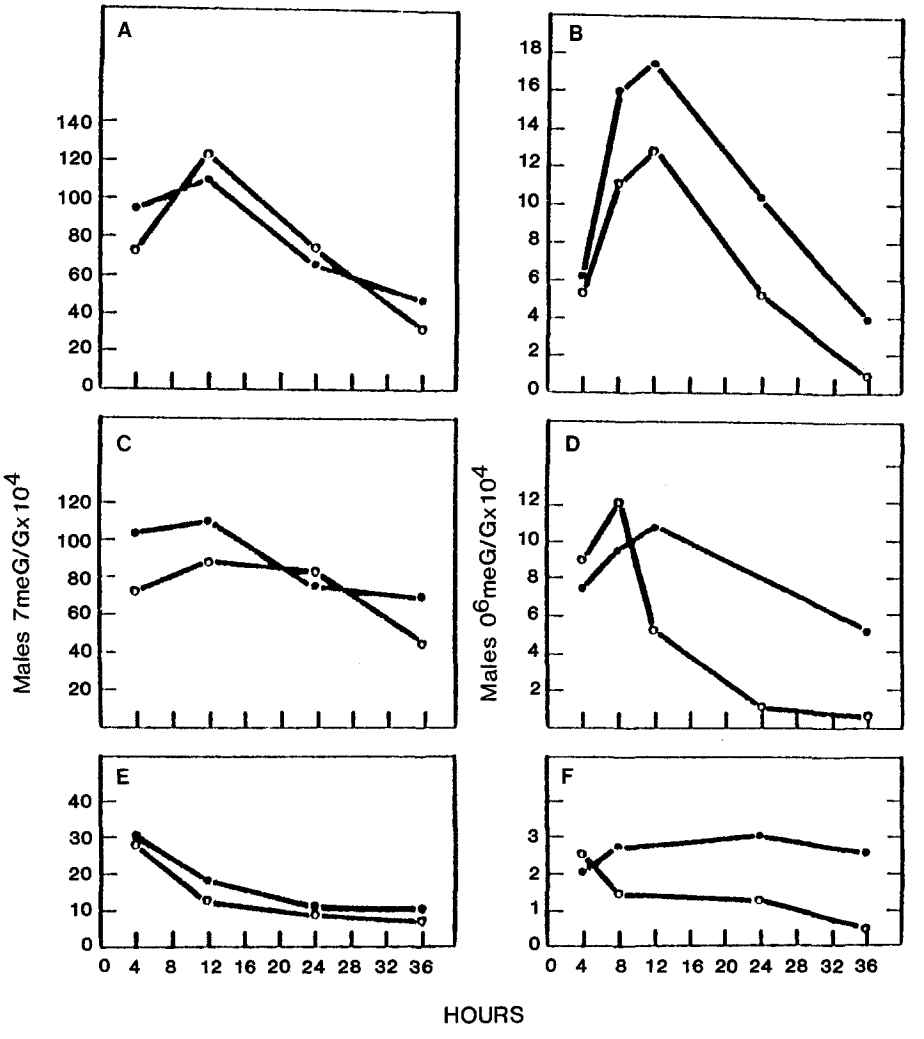

Figure 1. Time course of the formation and loss of 7-methylguanine and O^6-methylguanine from hepatic DNA of rats which had been maintained on an ethanol-containing (●) or control diet (○). The animals were injected at 0 hr with ^{14}C-labeled dimethylnitrosamine at doses of 25 mg/kg (A,B); 10 mg/kg (C,D), and 1.5 mg/kg (E,F). The purines were liberated from isolated DNA by acid hydrolysis and separated by chromatography on Sephandex G-10 columns. The concentrations of the alkylated bases were calculated from the initial specific activities of the DMN and the guanine concentrations were determined spectrophotometrically (27). Each point represents a pair of animals.

to 7meG for DNAs from the two groups of rats. The results, as depicted in Fig. 2, show that the ratio of O^6meG to 7meG increased from approximately 1 to 3-4 times that level in alcohol-fed animals compared to their controls during 4 to 36 hr post DMN treatment.

It should be noted that because of the expensive nature of these experiments, primarily due to the cost of the ^{14}C-labeled DMN and the need for using pair-fed animals, only two animals were used for each of the

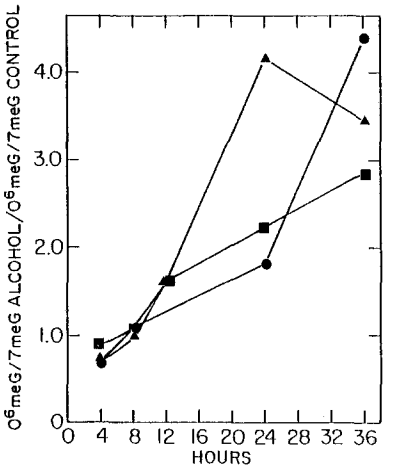

Figure 2. Relative persistence of O^6meG in hepatic DNA of alcohol-fed and control diet rats. The data are derived from the results presented in Fig. 1. DMN doses: 1.5 mg/kg (●); 10 mg/kg (▲); and 25 mg/kg (■).

time points shown in Fig. 1. However, the absence of the expected enhancing effect of ethanol on the initial levels of DNA alkylation was confirmed in an experiment in which four pairs of animals were treated with DMN at a dose of 1.5 mg/kg. The results presented in Table 1 show that the DNA alkylation levels observed in the alcohol-fed and control animals were not significantly different from each other. These results also are consistent with those reported by Schwarz et al. (28) who showed that pretreatment with ethanol (20% w/v) administered for two weeks in drinking water had no effect on DMN-induced alkylation measured 4 hr after DMN administration over a dose range of 0.25 mg/kg to 25 mg/kg. Furthermore, in our experiments the same general trend was observed for 7meG or O^6meG

TABLE 1.

Methylation of hepatic DNA by ^{14}C-DMN (3.7mCi/mmol) administered at a dose of 1.5 mg/kg to rats which had been pair-fed for 4 weeks with either an ethanol-containing (36% of total calories) or control diet. The animals were killed 4 hr post administration of the DMN, the hepatic DNA was isolated as described in the text and its ^{14}C counts were read in a scintilltion counter. Differences in the two treatments are not significantly different as determined by paired T-test (p 0.02).

^{14}C Counts, dpm/mg of DNA	
Alcohol Diet	Control Diet
748	681
619	691
509	682
609	607
Mean	
621	665

levels at the three different dose levels and the constancy of the differences at subsequent time intervals lend support to the conclusion that O^6meG persisted at higher levels with ethanol administration. Additional confirmation of this observation has been provided in subsequent studies from this laboratory (42,43) that are discussed in the next section.

DISCUSSION

Ethanol is known to cause a proliferation of endoplasmic reticulum (29,30) and to induce enzymes of the P-450 cytochrome system that are involved in the metabolism of a variety of carcinogens including nitrosamines (10,11). An increased metabolism of mitrosamines should lead to an increased formation of reactive intermediates and this is expected to increase DNA alkylation. However, contrary to this expectation the level of DNA alkylation was found not to be significantly different from their controls with chronic ethanol consumption. These observations are supported by the results of Schwarz et al. (28), discussed above, that did not find a significant difference in the DNA alkylation that could be ascribed to ethanol intake.

The level of DNA alkylation observed at any particular point in time is the result of two competing processes; the amount of reactive alkylated intermediates formed and the extent of their reaction with DNA, and loss or removal of such bases through the action of DNA repair enzymes. The effect of ethanol on reaction of alkylated intermediates with DNA is not known. However, while ethanol consumption was without an effect on DNA alkylation, interestingly enough, it did affect the rate of loss of alkylated species differently. Thus, although the levels of 7meG at subsequent time intervals were not significantly different in the two diet groups, we were surprised to find that consistently larger amounts of O^6meG persisted with ethanol intake. This surprise was due to the fact that a number of studies have shown that O-alkylated bases including O^6meG and O^4methylthymine are important DNA lesions that are involved in initiating mutagenesis and carcinogenesis (for example, see 19-24, 31-33). An increased persistence of O^6meG could, therefore, explain the increased incidence of cancers that are associated with chronic alcohol consumption. Experimental support for an increased mutagenicity consequent upon ethanol intake is provided by the observation that when microsomes from ethanol pretreated rats are used in the Ames bioassays, the mutagenicity of DMN is significantly increased (34,35). Furthermore, notwithstanding some contradictory reports, a number of studies have shown that there was an increase in chemically induced cancers with ethanol administration in experimental animals (for example, see 36-40).

The lack of ethanol effect on loss of 7meG implies that ethanol may not interfere with the repair process that is normally employed for the removal of 7meG from DNA. On the other hand, the persistence of O^6meG lesions indicates that ethanol interferes with the normal repair of such promutagenic/carcinogenic lesions.

A preliminary report of the above results was presented at the Conference of American Association for Cancer Research in 1981 (41). Further studies from this laboratory have upheld the above conclusions. Farinati et al. (42) observed that 71% of O^6meG persisted 36 hr after i.p. administration of 0.62 mCi/mmol of ^{14}C-DMN. Furthermore, the effect was specific for O^6meG since the removal of DNA adducts induced by acetylaminofluorene, which are repaired by an excision pathway, was unaffected. This and another study by Garro et al. (43) measured the levels of O^6meG repair enzyme (O^6meG transferase) and results obtained showed that indeed the levels of O^6meG transferase were depressed by chronic ethanol consumption. Feeding of the ethanol-containing Lieber-DeCarli diet (36% of total caloric intake given as ethanol) for 4 weeks reduced the transferase activity by about 40% relative to controls. However, it is not clear whether it is ethanol or its metabolites such as acetaldehyde that cause the depression and whether it is synthesis or activity of the transferase enzyme that is inhibited.

It may be pointed out that in contrast to our results, Belinsky et al. (44) observed that ethanol pretreatment for 3 weeks did not affect the persistence of O^6meG determined until 72 hr after administration of 4mg/kg of DMN. However, in their study the experimental rats were maintained on a diet of low caloric intake and also administered a lesser amount of ethanol compared to ours; for this reason it is difficult to attribute the lack of ethanol effect to any one factor but a low noninducing dose of ethanol or a caloric or nutritional deficiency may have contributed to the observed effect in that study.

On the other hand, a study by Kouros et al. (45) showed that even at 4 hr after treatment with an esophageal specific carcinogen, methylbenzylnitrosamine, prior administration of ethanol for 3-4 weeks significantly enhanced DNA methylation in the rat esophagus. This observation implies that the effect of ethanol on formation and persistence of O^6meG may be more clearly evident in a tissue such as esophagus which is more closely associated with alcohol-related cancers (46,47) and where the DNA repair may not be as efficient as in the liver.

ACKNOWLEDGMENTS

This research was supported by the Medical Research Service of the Veterans Administration, by the USPHS grant AA03508, by a National

Institutes of Environmental Health grant, and by a grant from the City University of New York PSC-CUNY Research Award Program.

REFERENCES

1. Hakulinen, T., Lehtimaki, L., Lehtonen, M., and Teppo, L. (1974) J. Natl. Cancer Inst., 52, 1711-1714.
2. Breslow, N.E., and Enstrom, J.E. (1974) J. Natl. Cancer Inst., 53, 631-639.
3. Feldman, J.G., Hazan, M., Nagarajen, M., and Kissin, B. (1975) Prev. Med., 4, 444-463.
4. Williams, R.R., and Horm, J.W. (1977) J. Natl. Cancer Inst., 58, 525-547.
5. Schottenfeld, D. (1979) Cancer, 43, 1962-1966.
6. Kono, S., and Ikeda, M. (1979) Br. J. Cancer, 40, 449-455.
7. Tuyns, A.J. (1979) Cancer Res., 39, 2840-2843.
8. Doll, R., and Peto, R. (1981) J. Natl. CAncer Inst., 66, 1191-1308.
9. Lieber, C.S. (1982) Medical Disorders of Alcoholism: Pathogenesis and Treatment, p. 11, Saunders, Philadelphia.
10. Gillette, J.R. (1976) Fed. Proc. 35, 1142-1147.
11. Miller, J.A. and Miller, E.C. (1977) Origins of Human Cancer, p. 605, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
12. Lawley, P.D. (1974) Mutat. Res. 23, 283-295.
13. Magee, P.M., Montesano, R., and Pressman, R. (1976) Chemical Carcinogens, p. 491, ACS monograph No. 173, American Chemical Society, Washington, D.C.
14. Margison, G.P., and O'Connor, P.J. (1979) Chemical Carcinogens, p. 111, CRC Press, West Palm Beach, Florida.
15. Margison, G.P., and Pegg, A.E. (1981) Proc. Natl. Acad. Sci. USA, 78, 861-865.
16. Singer, B., and Brent, T.P. (1981) Proc. Natl. Acad. Sci. USA, 78, 856-860.
17. Loveless, A. (1969) Nature (London), 223, 206-207.
18. Williams, L.D., and Shaw, B.R. (1987) Proc. Natl. Acad. Sci. USA, 84, 1779-1783.
19. Goth, R., and Rajewsky, M.F. (1974) Proc. Natl. Acad. Sci. USA, 71, 639-643.
20. Margison, G.P., and Kleihues, P. (1975) Biochem. J., 148, 521-525.
21. Newbold, R.F., Warren, W., Medcalf, A.S.C., and Amos, J. (1980) Nature (London), 283, 596-599.
22. Sklar, R., and Strauss, B. (1980) J. Mol. Biol. 143, 343-362.
23. Dodson, L.A., Foote, R.S., Mitra, S., and Masker, W.E. (1982) Proc. Natl. Acad. Sci., USA, 79, 7440-7444.
24. Singer, B. and Grunberger, D. (1983) Molecular Biology of Mutagens and Carcinogens, Plenum, New York.
25. Lieber, C.S., and DeCarli, L.M. (1970) Am. J. Clin. Nutr. 23, 474-478.
26. Lieber, C.S., and DeCarli, L.A. (1982) Alcohol. Clin. Exp. Res., 6, 523-531.
27. Montesano, R., Bresil, H., and Margison, G.P. (1979) Cancer Res., 39, 1798-1802.
28. Schwarz, M., Wiesbeck, G., Hummel, J., and Kunz, W. (1982) Carcinogenesis, 3, 1071-1075.
29. Iseri, O.A., Gottlieb, L.S., and Lieber, C.S. (1964) Fed. Proc., 23, 579.
30. Iseri, O.A., Lieber, C.S., and Gottlieb, L.S. (1966) Am. J. Path., 48, 535-555.
31. Singer, B., Spengler, S.J., Fraenkel-Conrat, H., and Kusmierek, J.T. (1986) Proc. Natl. Acad. Sci. USA, 83, 28-32.
32. Swenberg, J.A., Dyroff, M.C., Bedell, M.A., Popp, J.A., Huh, N., Kirstein, U., and Rajewsky, M.F. (1984) Proc. Natl. Acad. Sci. USA, 81, 1692-1695.

33. Doniger, J., Day, R.S., and DiPaolo, J.A. (1985) Proc. Natl. Acad. Sci. USA, 82, 421-425.
34. Garro, A.J., Seitz, H.K., and Lieber, C.S. (1981) Cancer Res., 41, 120-124.
35. Neis, J.M., te Brommelsroet, B.W.J., Van Gemert, P.J.L., Roelofs, H.M.J., and Henderson, P.Th. (1985) Arch. Toxicol., 57, 217-221.
36. Gibel, Von W. (1967) Arch. Geschwulstforsch., 30, 181-189.
37. Griciute, L., Castegnaro, M., Bereziat, J.-C. (1982) N-Nitroso Compounds: Occurrence and Biological Effects, p. 643, IARC Scientific Publication, Lyon, France.
38. Gabriel, G.N., Schrager, T.F., and Newberne, P.M. (1982) J. Natl. Cancer Inst., 68, 785-789.
39. McCoy, C.D., Hecht, S.S., and Furuya, K. (1986) Cancer Lett., 33, 151-159.
40. Mufti, S.I., Becker, G., and Sipes, I.G. (1988) Cancer Res., Submitted for publication.
41. Mufti, S.I., Lieber, C.S., and Garro, A.J. (1981) Proc. Am. Assoc. Cancer Res., 22, 58.
42. Farinati, F., Espina, N., Lieber, C.S., and Garro, A.J. (1984) Gastroenterology, 86, 1317.
43. Garro, A.J., Espina, N., Farinati, F., and Salvagnini, M. (1986) Alcohol. Clin. Exp. Res., 10, 73S-77S.
44. Belinsky, S.A., Bedell, M.A., and Swenberg, J.A. (1982) Carcinogenesis, 3, 1293-1297.
45. Kouros, M., Monch, W., Reiffer, F.J., and Dehnen, W. (1983) Carcinogenesis, 4, 1081-1084.
46. Rothman, K., Garfinkel, L., Keller, A.Z., Muir, C.S., and Schottenfeld, D. (1980) Prev. Med., 9, 174-179.
47. Tuyns, A.J. (1983) Int. J. Cancer, 32, 443-444.