in vivo *22*: 793-798 (2008)

Early Modification of *c-myc*, *Ha-ras* and *p53* Expressions by *N*-Methyl-*N*-nitrosourea

FERENC BUDÁN[1], TÍMEA VARJAS[1], GHODRATOLLAH NOWRASTEH[1], ZSUZSA VARGA[2], IMRE BONCZ[3], JÓZSEF CSEH[4], IDA PRANTNER[1], TIBOLD ANTAL[1], EMESE PÁZSIT[5], GYULA GŐBEL[6], MIKLÓS BAUER[6], TÜNDE GRACZA[7], PÁL PERJÉSI[8], ISTVÁN EMBER[1] and ZOLTÁN GYÖNGYI[1]

[1]*Department of Public Health,* [6]*Department of Otolaryngology Head and Neck Surgery,*
[7]*Mihály Pekár Medical and Life Science Library,*
[8]*Institute of Pharmaceutical Chemistry, Medical School, and*
[3]*Department of Health-Economy, Health Politic and Health Management,*
Faculty of Health Sciences, University of Pécs, H-7643 Pécs;
[2]*Country Hospital of Baranya, Department of Oncology, H-7623, Pécs;*
[4]*Szent György Country Hospital, Department of Oncology, H-8000, Székesfehérvár;*
[5]*Hospital And Clinic of Diósgyőr,Obstetrical Ward, H-3520, Miskolc, Hungary*

Abstract. *Methylnitrosourea (MNU) is a well-known pluripotent direct-acting carcinogen. Formation of MNU following incubation of various meats with additional nitrite under in vitro acidic conditions is possible. It is possible that many species, including humans, are exposed to carcinogenic MNU, generated in their alimentary tract. Previously, an animal model was developed by our research group to investigate the expression of three genes c-myc, Ha-ras and p53 as early molecular epidemiological biomarkers of carcinogenic exposure or carcinogenesis caused by DMBA (dimethylbenz[α]anthracene). The aim of this study was to investigate the early effect of MNU on the gene expression levels. MNU is a direct-acting carcinogen which spontaneously and rapidly degrades, so any effect on the gene expression is observed in 24 hours. Our results show the maximum effect in vivo on the gene expression at 12 hours after the MNU treatment; on the other hand, 24 hours after the treatment, the elevated gene expressions decreased in target organs (bone marrow, lung, lymph nodes). Our results correspond to "long-term" experiments of the carcinogenic effect of MNU in different target organs. Our findings suggest that MNU has an impact on the expression of c-myc, Ha-ras and p53 genes in 12 hours, especially in bone marrow. Overexpression of these genes occurs as an early biological effect of exposure to chemical carcinogens. According to our results, the high expression of these genes could indicate MNU exposure and these genes could take part in MNU-induced tumorigenesis.*

Correspondence to: Zoltán Gyöngyi, Department of Public Health, University Medical School of Pécs, H-7643 Pécs, Hungary. e-mail: zoltan.gyongyi@aok.pte.hu

Key Words: Gene expression, carcinogenesis, *ras*, *Ha-ras*, *p53*, *c-myc*, MNU, DMBA, mice.

According to data of Sen *et al*. formation of methylnitrosourea (MNU) following incubation of various meats with additional nitrite under *in vitro* acidic conditions is possible (1). MNU is a well-known pluripotent, direct-acting carcinogen (2, 3). Its structure is shown in Figure 1. It has been shown that MNU is able to induce cancer of various organs, mainly of the forestomach, lung and the central nervous system, different types of leukemia and lymphoma in a wide variety of animal species. It is possible that many species, including humans, are exposed to the carcinogenic effect of MNU generated in their gastrointestinal tract (4). Moreover when mice were administered an intraperitoneal injection of MNU, thymic lymphomas developed (5).

Both in the case of the formation of MNU in the gastrointestinal tract and experimentally administered MNU, mutations and amplification of *Ha-ras* have been shown (6). The question arises of when the first sign of the carcinogenic effect of MNU can be detected and whether overexpression of different key onco/supressor genes in the early steps of MNU-induced carcinogenesis can be detected?

Previously, an animal model was developed by our research group for investigating the expression of *c-myc, Ha-ras* and *p53* genes as early molecular epidemiological biomarkers of the effects of carcinogenesis and the exposure to carcinogenic agents (7-8). It is well-known that overexpression of these three genes signalizes the first steps of tumour formation and additional information about carcinogenesis can be gained *via*

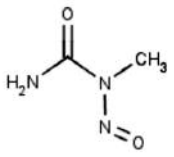

Figure 1. *Structure of MNU.*

the measurement of the expression of the *p53* gene in particular (9-13). In our earlier studies, we investigated the effect of dimethylbenz[α]anthracene (DMBA) on molecular epidemiological biomarkers (on CBA/Ca inbred mice). DMBA is also a pluripotent chemical carcinogen. It is activated by metabolic enzymes and acts as an initiator by causing point mutations in certain oncogenes and tumour suppressor genes (14). DMBA was found to induce overexpression of *Ha-ras* and *p53* genes 24-48 hours after *in vivo* treatment of mice (15). By the same method, we have examined here the expression of these three genes after intraperitonial administration of a direct acting alkylating agent, MNU, in mice.

Materials and Methods

Treatments. Six- to eight-week-old conventionally maintained, CBA/Ca inbred mice, with H-2^K haplotype (6-6 in each group, male and female) weighing 20-24 g were used for this experiment. Two groups were treated intraperitoneally (*i.p.*) with a single 30 mg/kg body weight dose of MNU (Sigma Aldrich, Budapest, Hungary) dissolved in 0.1 ml Salsol A (Teva Pharmaceutical Industries Ltd., Debrecen, Hungary). Control groups of mice treated *i.p.* with 0.1 ml Salsol A served as control.

Gene expression investigations. Four groups of mice were used, 6 males and 6 females in each. At 12 and 24 hours after the MNU or the Salsol A (control) treatment, the mice were autopsied by cervical dislocation and the liver, lungs, kidneys, thymus, spleen, lymph nodes and bone marrow were removed and 100 mg samples of each tissue were pooled according to groups. After homogenization of the organs, total cellular RNA was isolated using TRIZOL reagent (Invitrogen, Paisley, Scotland, UK). The concentration and quality of the RNA was assessed by absorption measurement of light at 260/280 nm wavelength. RNA of each sample (10 μg) was dot-blotted onto Hybond N+ nitrocellulose membranes (ECL kit; Amersham, Little Chalfont, UK) and hybridized with chemiluminescently labelled specific probes for *c-myc*, *p53* and *Ha-ras* (Professor J. Szeberényi; University of Pécs, Hungary) genes. The RNA isolation, hybridization and detection were performed according to the manufacturer's instructions. The signals were detected on X-ray films. The dots were evaluated by Quantiscan software (Biosoft, Cambridge, UK). Gene expression is reported as % relative to the level of the expression of *β-actin* control.

Results

The expression of mRNA of the *Ha-ras* gene is shown in treated and control groups, 12 and 24 hours after the treatment (Figure 2). Twelve hours after MNU treatment, the expression of *Ha-ras* gene was higher in lymph nodes, bone marrow and the lungs, while in the liver, spleen and thymus it was lower in comparison with that of the control groups. Twenty-four hours after the treatment, the expression level of the *Ha-ras* gene was lower in lymph nodes but higher in the bone marrow as compared to the control group (Figure 2).

As shown in Figure 3, the expression of the *p53* tumour suppressor gene was higher in treated groups at 12 hours after the treatment. Twelve hours after the MNU treatment, the expression level of the mRNA of *p53* gene was higher in liver, lung and bone marrow in comparison to the control, while in the spleen, kidneys and lymph nodes the expression of *p53* gene did not show any remarkable difference. Twenty-four hours after the treatment, the expression of the *p53* gene decreased strongly in kidneys, thymus and lymph nodes in treated groups. Interestingly, twenty-four hours after the treatment, in the bone marrow the expression of the *p53* gene completely ceased.

The expression of the *c-myc* gene at 12 hours after the MNU treatment was remarkably higher in lungs, lymph nodes and bone marrow but lower in the liver, and especially in the thymus compared to controls. Twenty-four hours after the treatment, the expression of the *c-myc* gene was diminished in both treated and untreated groups, with expression in treated groups being lower in all tissues than in the control groups (Figure 4).

Discussion

The molecular structure of MNU (Figure 1) explains its water solubility, which in addition to its small molecular size leads to the fast distribution of the molecule. The spontaneously degrading nature of MNU could explain our observation that a more enhanced effect was found 12 hours after its administration than later. On the basis of our results, where we could find early effect, one could assume that the molecule acts in its unaltered form and does not need to be metabolized. The elevation of the *Ha-ras* gene was observed both in bone marrow and in lymph nodes (16). Hence the *Ha-ras* gene is involved in the early phases of MNU-induced carcinogenesis.

As to the molecular mechanism of tumour induction by MNU, there are different explanations in the literature. Hoivik *et al.* were able to induce lymphomas in heterozygous null *p53* (+/–) mice with a single dose of MNU (17). It is possible that *p53* has a protective effect against the action of MNU. In our experiments, the elevation of *p53* expression was conspicuous in the bone marrow.

Reese *et al.* emphasized the importance of the formation of O^6-alkylguanine after administration of MNU. DNA alkyltransferase and *p53* could exert a protective effect (18). The mechanism of action of both of the alkylating agents (MNU and DMBA) involves their conversion to the active carbonium ion and subsequent methylation of

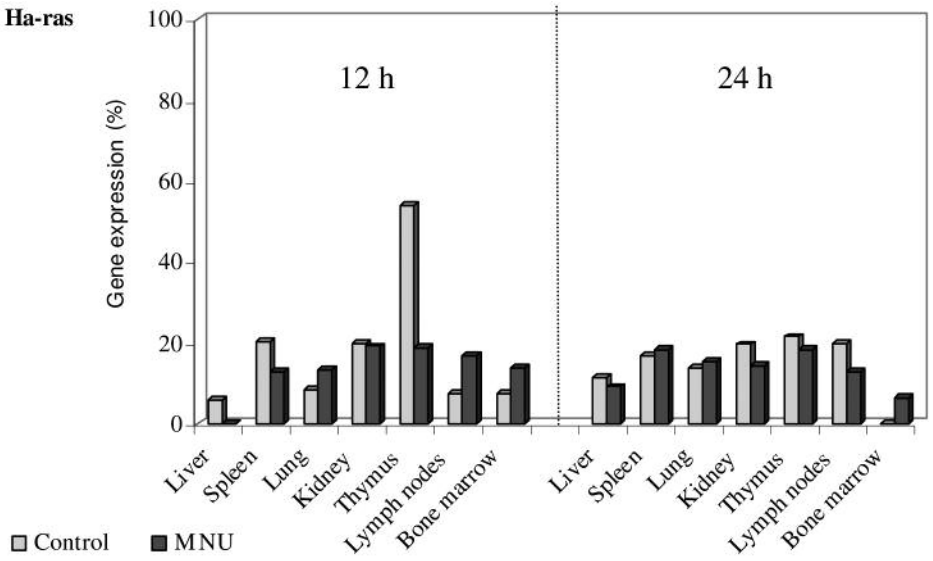

Figure 2. *Ha-ras gene expression pattern from the different tissues of CBA/CA mice at 12 and 24 hours after the treatment (the arbitrary unit is gene expression as % of β-actin).*

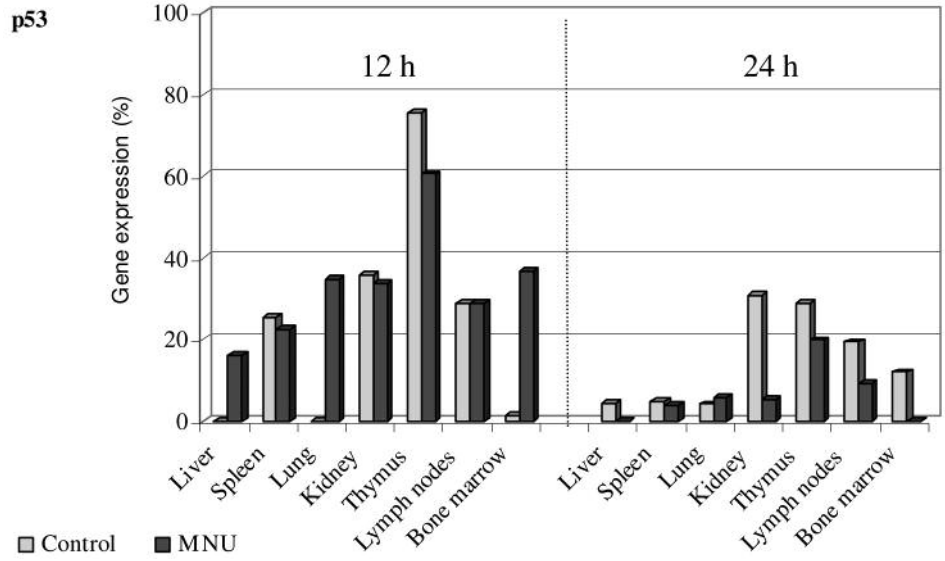

Figure 3. *p53 Gene expression pattern from the different tissues of CBA/CA mice at 12 and 24 hours after the treatment (the arbitrary unit is gene expression as % of β-actin).*

cellular macromolecules, usually DNA. MNU is a DNA alkylating agent by formation of O^6 methyl guanine (O^6mG) adducts (17-19).

Kang *et al*. found overexpression of the *c-myc* gene in lymphomas induced by MNU (20). Moreover, weekly treatment with MNU resulted in the increased incidence of tracheal tumours in hamsters (21). According to Seidel *et al*. T-cell leukaemia was induced in mice by MNU (22, 23). The remarkable elevation of *Ha-ras* protooncogene after 12 hours in lung, lymph nodes and bone marrow does correspond to the results of long-term (from weeks to years) experiments (20).

Talcott *et al*. showed in their experiment that MNU treatment significantly reduced the size of the thymus within one day. This fact corresponds to our earlier finding in the thymus. The expression of *Ha-ras* and *c-myc* genes decreased after MNU treatment (24). In our earlier studies,

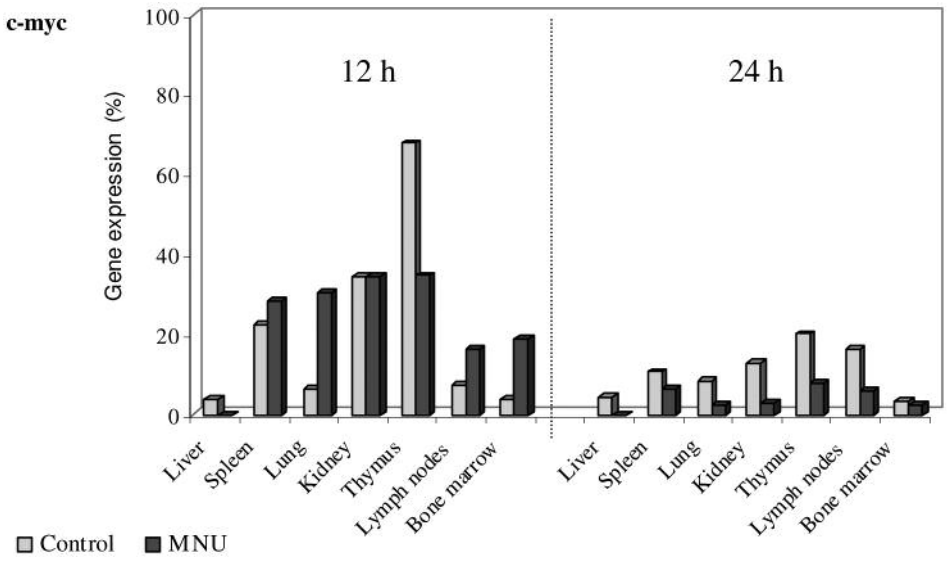

Figure 4. *c-myc Gene expression pattern from the different tissues of CBA/CA mice at 12 and 24 hours after the treatment (the arbitrary unit is gene expression as % of β-actin).*

CBA/Ca inbred mice were used in our onco/supressor gene expression model, but there are more susceptible mice strains available regarding the thymus effect of MNU treatment; in the future we are planning to experiment on AKR mice to gain more knowledge on the gene expression in the thymus induced by MNU treatment (15, 22).

Although MNU is direct acting and spontaneously degrades within an hour *in vitro*, and usually also *in vivo*, the effect on gene expression by 24 hours is close to the initial level (25). Our results 12 hours after the treatment imply that the expression of *p53* and *c-myc* correspond to those in the literature; while in the target organs of carcinogenesis: bone marrow, lymph nodes and lungs, the expression of *p53* increased (which may refer to DNA damage) and therefore the expression of *c-myc* in these organs consequently decreased. A negative feedback of *c-myc*-regulated *p53* expression is expected, which explains our previous finding 24 hours after treatment in lymph nodes and bone marrow, while the expression of *p53* decreased strongly under the control, although the direct effect of MNU should be carried through (26).

Indeed, DMBA is a metabolically activated carcinogen. The major metabolite of DMBA, is an ultimate carcinogen, 7-hydroxymethyl-12-methylbenz[α]anthracene (HMBA), which is a benzylic carbonium ion generated from an exceptionally reactive alkylating metabolite (27). In addition, an alkylating electrophilic mutagen and carcinogen is formed from HMBA, which is itself either an ultimate carcinogen or a direct precursor of an ultimate carcinogen, *i.e.* a benzylic carbonium ion. This corresponds to our earlier findings: the effect of DMBA on the gene expression can be monitored at 24 and 48 hours after the treatment, while metabolic activation delays the effect compared to the effect of MNU (16).

Regardless of the exact molecular mechanism of tumorigenesis by MNU, it is important to state that in the experiments mentioned above, a single initial dose was enough to initiate tumour formation. According to our results, MNU seems to have an impact on the expression of *c-myc*, *Ha-ras* and *p53* genes in 12 hours, especially in bone marrow. The high expression of these genes could indicate MNU exposure and that they take part in MNU-induced tumorigenesis. Therefore, the investigation of the early events after the administration of MNU could improve our knowledge about the first steps of tumour formation.

Acknowledgements

The authors express their special thanks to Zsuzsanna Bayer and Mónika Herczeg for valuable technical assistance.

References

1 Sen NP, Seaman SW, Burgess C, Baddoo PA and Weber D: Investigation on the possible formation of *N*-nitroso-*N*-methylurea by nitrosation of creatinine in model systems and in cured meats at gastric pH. J Agric Food Chem *48*: 5088-5096, 2000.

2 McCaffrey J and Hamilton JW: Comparison of effects of direct-acting DNA methylating and ethylating agents on inducible gene expression *in vivo*. Environ Mol Mutagen *23*: 164-170, 2006.

3 Lee JS, Park EH, Choe J and Chipman JK: N-methyl-N-nitrosourea (MNU) induces papillary thyroid tumours which lack *ras* gene mutations in the hermaphroditic fish *Rivulus marmoratus*. Terat Carc Mutagen *20*: 1-9, 1999.

4 Tudek B, Bird RP and Bruce WR: Foci of aberrant crypts in the colons of mice and rats exposed to carcinogens associated with foods. Cancer Res *49*: 1236-1240, 1989.
5 Benavides F, Gomez G, Venables-Griffith A, Lambertz I, Flores M, Angel JM, Fuchs-Young R, Richie ER and Conti CJ: Differential susceptibility to chemically induced thymic lymphomas in SENCARB and SSIN inbred mice. Mol Carcinog *45*: 543-548, 2006.
6 Quintanilla M, Brown K, Ramsden M and Balmain A: Carcinogen-specific mutation and amplification of *Ha-ras* during mouse skin carcinogenesis. Nature *322*: 78-80, 1986.
7 Gyöngyi Z, Ember I, Kiss I and Varga C: Changes in expression of onco- and suppressor genes in peripheral leukocytes as potential biomarkers of chemical carcinogenesis. Anticancer Res *21*: 3377-3380, 2001.
8 Gyöngyi Z, Grama L, Nádasi E, Sándor J, Németh A, Varga C, Kiss I and Ember I: Flow cytometric analysis of DMBA-induced early *in vivo* ras expression. In Vivo *16*: 323-326, 2002.
9 Ember I, Kiss I, Gombkötő G, Müller E and Szeremi M: Oncogene and supressor gene expression as a biomarker for ethylene oxide exposure. Cancer Detect Prev *22*: 241-245, 1998.
10 Kozma L, Kiss I, Nagy A, Szakall S and Ember I: Investigation of *c-myc* and *K-ras* amplification in renal clear cell adenocarcinoma. Cancer Lett *111*: 127-131, 1997.
11 Pelling JC, Ernst SM, Strawhecker JM, Johnson JA, Nairn RS and Slaga TJ: Elevated expression of *Ha-ras* is an early event in two-stage carcinogenesis in SENCAR mice. Carcinogenesis *9*: 1599-1602, 1986.
12 Pelling JC, Fisher SM, Neades R, Strawecker J and Schweicker L: Elevated expression and point mutation of the *Ha-ras* protooncogene in mouse skin tumors promoted by benzyl peroxide and other promoting agents. Carcinogenesis *8*: 1481-1484, 1987.
13 Walker DR, Bond JP, Tarone RE, Harris CC, Makalowski W, Boguski MS and Greenblatt MS: Evolutionary conservation and somatic mutation hotspot maps of *p53*: correlation with p53 protein structural and functional features. Oncogene *18*: 211-218, 1999.
14 Ember I, Kiss I and Pusztai Z: Effect of 7,12-dimethylbenz(a)anthracene on onco/suppressor gene action *in vivo*: a short-term experiment. Anticancer Res *18*: 445-447, 1998.
15 Szanyi I, Lujber L, Gerlinger I, Pytel J, Bauer M, Csejtey A, Szele E, Gombos K, Kiss I, Seredenin S, Yarkova M and Ember I: *In vivo* effects of afobazole (2-mercaptobenzimidazole derivative) on the 7,12-dimethylbenz[alpha]anthracene-induced oncogene and suppressor gene expression. In Vivo *21*: 1059-1063, 2007.
16 Perjési P, Bayer Z and Ember I: Effect of E-2-(4'-methoxybenzylidene)-1-benzosuberone on the 7,12-dimethylbenz[a]anthracene-induced onco/suppressor gene action *in vivo* I: A 48-hour experiment. Anticancer Res *20*: 475-482, 2000.
17 Hoivik DJ, Allen JS, Wall HG, Nold JB, Miller RT and Santostefano MJ: Studies evaluating the utility of *N*-methyl-*N*-nitrosourea as a positive control in carcinogenicity studies in the p53 +/– mouse. Int J Toxicol *24*: 349-356, 2005.
18 Reese JS, Allay E and Gerson SL: Overexpression of human O^6-alkylguanine DNA alkyltransferase (AGT) prevents MNU induced lymphomas in heterozygous *p53*-deficient mice. Oncogene *20*: 5258-5263, 2001.
19 Weinkam RJ and Dolan ME: Intracellular activation of cytotoxic agents: kinetic models for methylnitrosoureas and *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine in cell culture. Chem Res Toxicol *2*: 157-161, 1989.
20 Kang HM, Jang JJ, Langford C, Shin SH, Park SY and Chung YJ: DNA copy number alterations and expression of relevant genes in mouse thymic lymphomas induced by gamma-irradiation and *N*-methyl-*N*-nitrosourea. Cancer Genet Cytogenet *166*: 27-35, 2006.
21 Becci PJ, Thompson HJ, Grubbs CJ and Moon RC: Histogenesis and dose dependence of *N*-methyl-*N*-nitrosourea-induced carcinoma in a localized area of the hamster trachea. J Natl Cancer Inst *64*: 1135-1140, 1980.
22 Seidel HJ and Kreja L: Leukemia induction by methylnitrosourea (MNU) in selected mouse strains. Effects of MNU on hemopoietic stem cells, the immune system and natural killer cells. J Cancer Res Clin Oncol *108*: 214-220, 1984.
23 Seidel HJ and Kreja L: T-cell subsets in the murine thymus during chemically induced leukemogenesis. Folia Histochem Cytobiol *24*: 27-31, 1986.
24 Talcott PA, Exon JH and Koller LD: The effects of methylnitrosourea (MNU) on natural killer (NK) cell cytotoxicity and cytokine production in rats. Carcinogenesis *5*: 829-34, 1990.
25 Weinkam RJ and Keenan TH: A method for measuring the lifetime of chemically and metabolically generated electrophilic species. Chem Biol Interact *46*: 255-264, 1983.
26 Ember I, Gyöngyi Z, Kiss I, Ghodratollah N and Arany I: The possible relationship between onco/suppressor gene expression and carcinogen exposure *in vivo*: evaluation of a potential biomarker in preventive and predictive medicine. Anticancer Res *22*: 2109-2116, 2002.
27 Flesher JW, Horn J and Lehner AF: 7-Sulfooxymethyl-12-methylbenz[a]anthracene is an exceptionally reactive electrophilic mutagen and ultimate carcinogen. Biochem Biophys Res Commun *231*: 144-148, 1997.

Received April 14, 2008
Revised June 24, 2008
Accepted July 18, 2008