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Genotoxic evaluation of the antimalarial drugs artemisinin and artesunate in human HepG2 cells and effects on CASP3 and SOD1 gene expressions

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ABSTRACT. The malaria treatment recommended by the World Health Organization involves medicines derived from artemisinin, an active compound extracted from the plant *Artemisia annua*, and some of its derivatives, such as artesunate. Considering the lack of data regarding the genotoxic effects of these compounds in human cells, the objective of this study was to evaluate the cytotoxicity and genotoxicity, and expressions of the CASP3 and SOD1 genes in a cultured human hepatocellular liver carcinoma cell line (HepG2 cells)

treated with artemisinin and artesunate. We tested concentrations of 2.5, 5, 7.5, 10, and 20 μg/mL of both substances with a resazurin cytotoxicity assay, and the concentrations used in the genotoxicity experiments (2.5, 5, and 10 μg/mL) and gene expression analysis (5 μg/mL) were determined. The results of the comet assay in cells treated with artemisinin and artesunate showed a significant dose-dependent increase ($P < 0.001$) in the number of cells with DNA damage at all concentrations tested. However, the gene expression analysis revealed no significant change in expression of CASP3 or SOD1. Our data showed that although artemisinin and artesunate exhibited genotoxic effects in cultured HepG2 cells, they did not significantly alter expression of the CASP3 and SOD1 genes at the doses tested.

Key words: Artemisinin; Artesunate; HepG2 cells; Gene expression; Comet assay; CASP3 and SOD1 genes

INTRODUCTION

Many plant extracts and their active components have been described and utilized as therapeutic agents (Verma and Singh, 2008); however, some plants also synthesize toxic substances as a primary defense against hordes of bacteria, fungi, insects, and other predators. Therefore, it is important to assess the genotoxic effects and changes in gene expression caused by medicinal extracts and their isolated compounds.

Artemisinin, a natural sesquiterpene lactone isolated from the plant *Artemisia annua*, is a widely used drug for treating resistant strains of malaria (Efferth et al., 2001). Artesunate is a semisynthetic derivative of artemisinin that has been accepted as a treatment for malaria (Price, 2000). Artemisinin and its derivative compounds are present in all five of the combined therapies recommended by the World Health Organization (WHO) for the treatment of uncomplicated falciparum malaria (WHO, 2010).

Some *in vitro* studies using cancer cell lines suggested that artemisinin and some of its derivatives have cytotoxic effects, alter the cell cycle, and induce apoptosis (Hou et al., 2008; Li et al., 2008; Youns et al., 2009; Zhang et al., 2010). These compounds may present cytotoxic activity in mammal cell proliferation (Efferth et al., 2001; Disbrow et al., 2005), and it has been suggested that the toxicity is related to high intracellular concentrations of iron (Smith et al., 1997; Singh and Lai, 2001).

Other studies have indicated that the mechanism of action of antimalarial drugs, including artemisinin and its derivatives, causes damage to membrane structures, which can directly influence normal cell functioning and initiate a series of biochemical reactions resulting in cell death (Olliaro and Taylor, 2004; Sato et al., 2007). In addition, it has been shown that endoperoxides of artemisinin trigger the production of reactive oxygen species (ROS), which has also been implicated in the mechanism of cytotoxicity (Disbrow et al., 2005). Embryotoxicity has been observed in rats and rabbits (Clark et al., 2004; Longo et al., 2006), and neurotoxicity has been observed both *in vitro* and *in vivo* (Schmuck et al., 2002).

Reports on mammalian cell genotoxicity are scarce for artesunate. Aquino et al. (2011) reported genotoxic effects of artesunate in liver cells of mice at low doses and clastogenic effects at high doses in bone marrow cells of the same animals. Li et al. (2008) and Mota et al. (2011) reported genotoxic and cytotoxic effects of artesunate *in vitro* in Chinese hamster ovary cells and in human peripheral lymphocytes, respectively. To the best of our knowledge, no genotoxic studies on mammalian cells of artemisinin and no genotoxic studies on metabolizing cells of artemisinin and artesunate have been carried out.

Cell death is obligatory for harmonious cell life in animals for the removal of infected, injured, or cancerous cells, and caspases are usually involved in this apoptotic process (Denault and Salvesen, 2002). Considering that ROS play important roles in the pathogenesis of many diseases, and that superoxide dismutases (SODs) are the major antioxidant defense systems against ROS (Fukai and Ushio-Fukai, 2011), it is important to evaluate expression of these genes when cells are exposed to new chemicals in order to better evaluate the cytotoxicity of these compounds.

Because artemisinin and artesunate are widely used drugs, with more than 100 million doses administered annually (WHO, 2010), it is essential to develop studies examining their genotoxic potential and to determine the molecular mechanisms of their cytotoxicity in order to evaluate their safe and effective use for malaria and their potential use in cancer treatment. By exposing human hepatocellular liver carcinoma (HepG2) cells to artemisinin and artesunate *in vitro*, this study evaluated both the cytotoxic and genotoxic effects of these compounds on mammalian DNA, as well as expressions of the caspase 3 (CASP3) and SOD1 genes to obtain more information about its genotoxic mechanism.

MATERIAL AND METHODS

Artemisinin and artesunate

The leaves of *A. annu*a L. were collected in the experimental field of medicinal plants of Multidisciplinary Research Chemical, Biological and Agricultural Center (CPQBA) - UNICAMP - in the town of Paulínia, SP. A voucher specimen was deposited in the herbarium of CPQBA (CPQBA 12.46). Leaves (5000 g) of *A. annua* were oven dried at 40°C, pressed, and extracted by maceration with 20 L hexane for 2 days. The macerated sample was filtered and concentrated under reduced pressure, providing 92 g crude extract hexane (1.84% yield). The artemisinin purification was conducted in chromatography columns using silica gel 60 230-400 mesh (0.040-0.063 mm particulates). Elution of sample components was achieved by incorporating the material with a mixture of n-hexane:ethyl acetate (9:1). The same mobile phase was used to run the column and the successive fractions obtained were monitored by thin layer chromatography. After purification of the sample, crystallization of the artemisinin was performed with selected solvents (Cafferata et al., 2009). Colorless crystals, with the typical characteristic of long needles, were obtained for a total of 16.4 g artemisinin (0.33% yield). The analysis was confirmed by nuclear magnetic resonance, whose spectra were obtained in a Bruker device DPX 400 MHz, operating at 400 MHz for [^{1}H] and at 100 MHz to [^{13}C]. The samples were dissolved in deuterated chloroform ($CDCl_3$, Sigma-Aldrich Co.).

Dihydroartemisinin (DHA) was prepared from artemisinin using a routine proce-

dure (Brossi et al., 1988). The chloride acid RCOCl was prepared from the corresponding carboxylic acids by heating with thionyl chloride at 55°C for 3 h and reacting with DHA in the presence of triethylamine in dry dichloromethane at 0°C for 2 h to furnish ester derivatives in 49-58% yields. Artesunate was prepared from DHA by reacting it with succinic acid anhydride in basic medium. Pyridine was used as the solvent, sodium bicarbonate as the base, and *N*,*N*-dimethylaminopyridine and triethylamine in 1,2-dichloroethane were used as catalysts, with yields of up to 100%. The mixture was stirred for up to 9 h to obtain artesunate in quantitative yield. The product was further re-crystallized from dichloromethane. Alpha-artesunate was exclusively formed (melting point = 135°-137°C). The purity of both compounds was estimated at 99% by both high performance liquid chromatography and spectral data analysis.

Cell line

The human hepatoma cell line HepG2 used in our study was obtained from the Cell Bank of Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil. Cells were cultivated in minimum essential media (Gibco) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (Gibco), at 37°C, 5% CO_2, and 95% relative humidity.

Cytotoxicity evaluation: resazurin-based assay

Since 1995, several studies have used resazurin as an indicator of cell viability (Yajko et al., 1995). In our experiments, we tested artemisinin and artesunate at concentrations chosen according to the available literature: 2.5, 5, 7.5, 10, and 20 µg/mL. The tests were done in biological duplicate with three repetitions each. Approximately 5 x 10^4 cells were seeded in each well of a 24-well microplate. After 24 h stabilization, cells were treated with the concentrations of artemisinin or artesunate described above, as well as with 2 µg/mL doxorubicin as a positive control, for 24 h. At the end of this period, the medium was removed, cells were washed with phosphate-buffered saline, and cells were incubated with 60 µM resazurin (Acros Organics) for 3 h. Fluorescence was measured with VICTOR 3 (Perkin Elmer) at 530-560 nm of excitation and a 580-600-nm emission range.

Comet assay

The comet test was carried out based on conditions described in Tice et al. (2000). One million cells were seeded for each experimental flask and, after 24 h stabilization, they were treated with artemisinin or artesunate at concentrations of 2.5, 5, and 10 µg/mL for 3 h. The positive control concentration chosen was 2 µg/mL, since it requires metabolization to exert genotoxic effects. After this, cells were trypsinized (500 µL 0.1% trypsin-EDTA, 37°C), the cell suspension was centrifuged (5 min, 1000 rpm), and the pellet was resuspended in 500 µL culture medium. Next, 120 µL low melting point agarose (0.5%) was added to the cells, this mixture was deposited on pregelatinized slides (1.5% normal melting point agarose), and placed in lysis solution for at least 1 h. After denaturation (20 min) and alkaline electrophoresis (25 V, 300 mA, 40 min), the slides were neutralized, fixed, and kept refrigerated until analysis. The slides were stained with ethidium bromide

and were analyzed visually (Kobayashi, 1995) under a fluorescence microscope (excitation filter of 420-490 nm and emission filter of 520 nm) at 400X magnification. The comets were classified as follows: class 0, nucleus without tail; class 1, nucleus with tail less than the diameter of the nucleus; class 2, nucleus with tail 1 to 2 times the diameter of the nucleus; class 3, nucleus with tail more than 2 times the diameter of the nucleus. Experiments were performed in triplicate, and 300 cells were analyzed per treatment. Determination of cell viability was carried out by the trypan blue exclusion method, where only treatments with greater than 80% viability were considered.

Quantitative real-time polymerase chain reaction (qRT-PCR)

For analysis of gene expression, 10^6 cells were seeded in each experimental culture flask. After 24 h stabilization, cells were treated with 5 μg/mL artemisin or artesunate for 12 h. Experiments were performed in duplicate.

Total RNA extraction was performed using the Trizol LS reagent (Invitrogen) according to manufacturer instructions. DNAse I (Invitrogen) treatment was also performed according to manufacturer instructions. RNA quality was verified on agarose gel (28S and 18S rRNA pattern of bands) and by the A_{260}/A_{280} ratio. The cDNA was synthesized with 10 pmol/mL oligo-dT primer and M-MLV reverse transcriptase (Invitrogen), and stored at -80°C until use in qRT-PCR experiments. qRT-PCR runs were performed in an Engine Opticon detection system (Bio-Rad) using the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen) under the following conditions: 50°C for 1 min, 95°C for 3 min, 35 cycles of 95°C for 20 s each, 60°C for 30 s, and 72°C for 20 s. The melting curve varied from 50 to 95°C, ready every 0.5°C. Data were normalized with the GAPDH primer (Sugaya et al., 2005), which was amplified in each run of PCR. The primer sequence of SOD1 was manufactured according to Ding et al. (2004) and the primer sequence of CASP3 was designed using the IDT tool available at http://www.idtdna.com/Scitools/Applications/Primerquest: forward 5'- GTGCTACAATGCCCCTGGAT-3' and reverse 5'-GCCCATTCATTTATTGCTTTCC-3'.

Statistical analysis

After verifying for normal distribution, data obtained from cytotoxicity and single cell gel electrophoresis assays were submitted to one-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test with the GraphPad Instat® software (version 3.01). Results were considered to be statistically significant at $P < 0.05$. Gene expression levels investigated by qRT-PCR were determined according to the Pfaffl (2001) method using the REST-384 software (Pfaffl et al., 2002).

RESULTS

Cytotoxicity assay (resazurin)

Results of duplicate resazurin tests indicated that the 7.5, 10, and 20 μg/mL concentrations of artemisinin and artesunate were cytotoxic (Figure 1). Based on these data,

we chose concentrations of 2.5, 5, and 10 µg/mL for the comet assay, and a concentration of 5 µg/mL for the gene expression analysis.

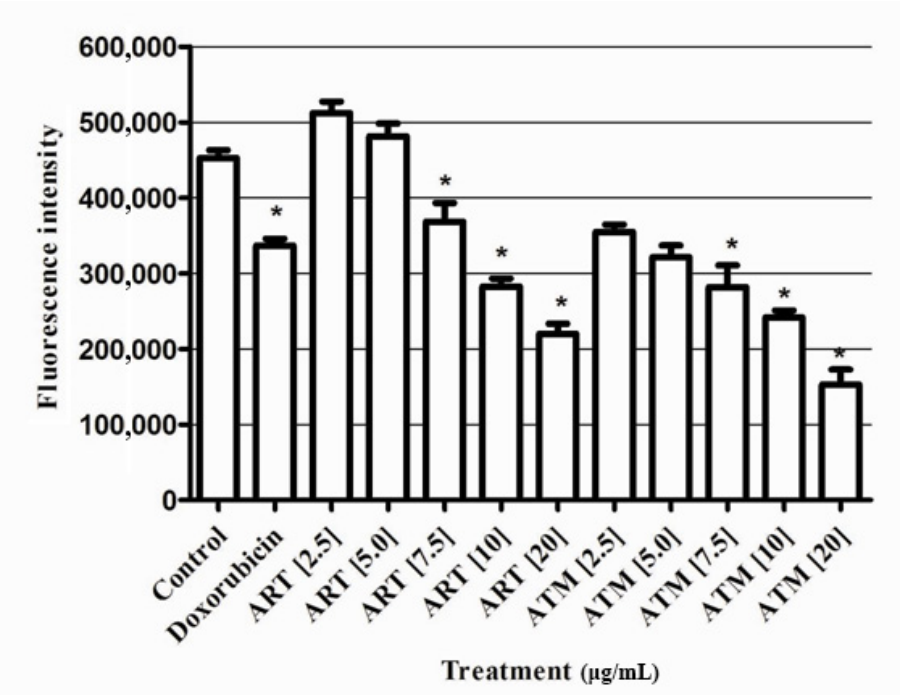

Figure 1. Cytotoxicity of artesunate (ART) and artemisinin (ATM) by the resazurin cytotoxicity assay. *Significantly different from the control ($P < 0.001$).

Comet assay

Under the experimental conditions, groups treated with both artemisinin and artesunate had more cells with DNA damage and higher damage scores relative to the negative control group at all three concentrations tested ($P < 0.001$) (Table 1). For both compounds, the majority of cells that suffered DNA damage showed light to medium damage (classes 1 and 2), with a minority showing severe damage (class 3). In general, artemisinin produced a greater number of cells with DNA damage classes 2 and 3 than did artesunate.

Analysis of expression levels of the CASP3 and SOD1 genes

According to the gene expression analysis, all groups maintained RNA integrity with satisfactory quantity and purity. The qRT-PCR analysis was used to detect changes in expressions of the two studied genes. Expression of CASP3 was slightly increased relative to controls. On the other hand, there was an increase in the expression of SOD1 in cells treated with artemisinin and a decrease in cells treated with artesunate. Nevertheless, the observed differences were not statistically significant (Figure 2).

Table 1. DNA migration in the comet assay for the assessment of genotoxicity of artemisinin and artesunate in HepG2 cells (after 3-h treatment).

Treatments	Total[1]	Comet class				Scores
		0	1	2	3	
Control	9	91	8	1	0	10
	11	89	9	2	0	13
	13	87	10	2	1	17
	11 ± 2.0					13.3 ± 3.5
Artemisinin (2.5 µg/mL)	93	7	46	42	5	145
	90	10	46	37	7	141
	92	8	45	40	7	146
	91.6 ± 1.5*					144 ± 2.6*
Artemisinin (5 µg/mL)	90	10	42	35	13	151
	95	5	48	38	9	151
	91	9	41	39	11	152
	92 ± 2.6*					151.3 ± 0.5*
Artemisinin (10 µg/mL)	94	6	42	33	19	165
	96	4	46	37	13	159
	97	3	38	36	23	179
	95.6 ± 1.5*					167.6 ± 10*
Artesunate (2.5 µg/mL)	52	48	40	12	0	64
	62	38	48	14	0	76
	70	30	53	16	1	88
	61.3 ± 9.0*					76 ± 12*
Artesunate (5 µg/mL)	70	30	53	14	3	90
	64	36	38	23	3	93
	68	32	46	20	2	92
	67.3 ± 3.0*					91.6 ± 1.5*
Artesunate (10 µg/mL)	92	8	41	34	17	160
	94	6	31	43	20	177
	98	2	29	53	16	183
	94.6 ± 3.0*					173.3 ± 11.9*
Doxorubicin (2 µg/mL)	95	5	15	39	41	166
	91	9	29	46	16	169
	92	8	25	52	15	174
	91 ± 4.5*					169.6 ± 4.0*

*Significantly different from the negative control ($P < 0.001$). [1]Total number of damaged cells (classes 1, 2, and 3).

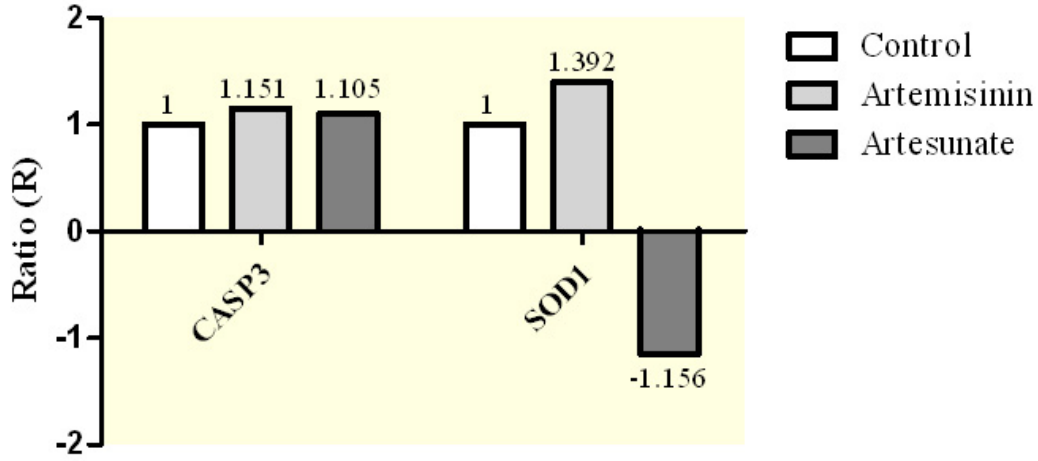

Figure 2. Relative expression of mRNA for CASP3 and SOD1, quantified by real-time PCR in HepG2 cell line.

DISCUSSION

Many medicines and environmental factors can cause DNA damage, and experiments analyzing the consequences of exposure to harmful agents are of fundamental importance for the development of medicines and environmental health research. Thus, with respect to artemisinin and artesunate, the presence of a safe balance between their therapeutic and toxicological effects should be verified.

A resazurin cytotoxicity assay was performed to determine the concentrations of artemisinin and artesunate to be tested in the present study. This assay is one of the most widely used methods and is the most sensitive test for detecting *in vitro* cytotoxicity (Fotakis and Timbrell, 2006). One of the advantages of this test is that it does not only assess cell death, but can also indicate inhibited cell growth, i.e., cytostatic effect. Our results showed that concentrations of 7.5, 10, and 20 mg/mL artemisinin and artesunate were cytotoxic to HepG2 cells after 24 h of exposure. Artesunate-induced changes in cell cycle kinetics were observed previously. Veerasubramanian et al. (2006) reported that artesunate, at concentrations lower than those used in the present study, could reduce the proliferation of cultured lymphocytes. Zhou and Feng (2005) reported that artesunate blocked the cell cycle during the G0/G1 phase in vascular smooth muscle cells from the aortas of mice. Electron microscopy of Panc-1, BxPC-3, and CFPAC-1 pancreatic cancer cells treated with artesunate revealed organelle destruction and internal disorganization in mitochondria, i.e., selective cytotoxic activity (Du et al., 2010).

In the present study, because the concentrations of 2.5 and 5 mg/mL artemisinin and artesunate were not cytotoxic to HepG2 cells, we performed the comet assay to verify whether these concentrations caused DNA damage. Results showed that all three concentrations tested of artemisinin and artesunate caused damage in the genetic material of HepG2 cells ($P < 0.001$), predominantly resulting in damage classes 1 and 2.

Reports on mammalian cell genotoxicity are scarce for artesunate, and are entirely lacking for artemisinin. Li et al. (2008) analyzed Chinese hamster ovary (CHO-9) and hamster lung fibroblast (V79-2) cell lines with the comet assay and verified that, *in vitro*, artesunate induced DNA damage and apoptosis. These authors also concluded that in mammalian cells, artesunate generated damage through direct or indirect oxidative stress, resulting in DNA strand breakage. Mota et al. (2011) also reported the genotoxicity of artesunate in human peripheral lymphocytes. The present study confirmed such genotoxicity *in vitro* for human metabolizing cells, and further studies are necessary to determine whether the generation of ROS could be involved in the genotoxicity of artesunate. Artesunate also showed genotoxic effects in peripheral blood leukocytes and liver cells of mice *in vivo* (Aquino et al., 2011). Our findings also agree with authors who assessed the genotoxic effects of other sesquiterpenes (Burin et al., 2001; Canalle et al., 2001; Al-Zubairi et al., 2010). In addition to causing DNA damage, the cytotoxicity of these substances, especially artemisinin, is due in part to the decreased expression of topoisomerase 2 (Youns et al., 2009), which is essential for cell proliferation and is expressed in dividing cells (Toyoda et al., 2008).

Using the MTT test, Hou et al. (2008) observed a significant reduction in cell viability in HepG2 cells treated with artemisinin and DHA (1-100 mM), with the percentage of viable cells ranging from 84.7 to 15.5% ($P < 0.01$) after 48 h of exposure. Also in this study, treating HepG2 cells with artemisinin at 10, 25, and 50 mM resulted in greater numbers of G1 phase cells (67.41, 70.72, and 69.21%, respectively), in comparison with untreated cells.

Apoptosis is a key regulator of physiological growth control and tissue homeostasis regulation. The methods currently used in clinical oncology and chemotherapy or immunotherapy to bring about the death of tumor cells involve activating apoptosis signal transduction. Hou et al. (2008) reported that artemisinin and artesunate exerted the greatest cytotoxicity on HepG2, inhibiting cell proliferation and inducing apoptosis by activating the CASP3 gene. Other authors also observed similar effects for both of these compounds (Li et al., 2008; Youns et al., 2009; Du et al., 2010; Zhang et al., 2010). We analyzed expression of the CASP3 gene in HepG2 cells after exposure to artemisinin and artesunate because it is the final common pathway of almost all apoptotic pathways. Both compounds caused a slight, although not statistically significant, increase in the expression of this gene, which may suggest that they alter the cell cycle but do not induce apoptosis (Figure 2). This non-significant increase in CASP3 expression may also be due to exposure time (12 h). Youns et al. (2009) observed increased CASP3 expression in experiments with artemisinin, although cells were treated for 6 h.

Due to artemisinin and the relationship of artesunate with oxidative stress induction (Michaelis et al., 2010), we chose to study expression of Cu/Zn SOD1. SODs catalyze the dismutation of the superoxide radical O_2 into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2), and thus provide an important defense against the toxicity of superoxide radicals. Our results showed that, compared to the control group, SOD1 expression increased in cells treated with artemisinin and decreased in those treated with artesunate, although these differences were not statistically significant. In a microarray analysis, Youns et al. (2009) also observed a slight decrease in expression of SOD1 after treating pancreatic cells with artesunate. Since only artemisinin treatment increased SOD1 expression, it is possible that this compound has a greater potential for generating oxidative stress than does artesunate. On the other hand, according to Effert et al. (2003) and Effert and Volm (2005), tumor cells can also present resistance to artesunate due to induced expression of antioxidant genes. This hypothesis should be further investigated.

In conclusion, our results indicated that artemisinin and artesunate are potent genotoxic compounds in human HepG2 cells and that a concentration of 5 μg/mL of these compounds was insufficient to produce significant alterations in CASP3 and SOD1 expressions. Therefore, based on our genotoxicity findings, caution regarding the intake of artemisinin and artesunate is necessary, since both may result in an increased risk of carcinogenesis.

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