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BIOPHYSICS OF COMPLEX SYSTEMS

# The Effect of Changes in CYP2E1 Activity in the Liver on Toxicity and Carcinogenicity of Diethylnitrosamine in Mice[1]

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**Abstract**—In this work, the biological effects of diethylnitrosamine (DENA) have been studied under controlled conditions of its metabolism in mice of different ages. The results indicate that the general toxic and hepatocarcinogenic effects of DENA are mostly due to the parent compound, whereas the alkylating metabolites cause hepatic cell damage. Our findings cast doubt on the conventional understanding of the exclusive role of mutagenic activation in the carcinogenic action of chemicals.

*Keywords*: suckling mice, diethylnitrosamine, metabolism, carcinogenicity, toxicity

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Diethyl- and dimethyl nitrosamines (DENA and DMNA) are widely used in experimental oncology to induce tumors in the liver, kidneys, and other organs of experimental animals. Endogenously, these compounds have been shown to undergo enzymatic dealkylation and denitrosation with subsequent formation of highly active low-molecular-weight metabolites capable of alkylating and thus damaging cellular macromolecules (including DNA) [1–5]. It was generally accepted that the bacteria that lack the respective nitrosamine-activating enzymes are insensitive to the action of nitrosocompounds [2]. Cytochrome P450 2E1 (CYP2E1) is one of the major enzymes involved in metabolic biotransformations of DENA (both deethylation and denitrosation). This enzyme is constitutively expressed in the liver and metabolizes various low-molecular-weight biogenic amines and alcohols as well as other nitrosamines [6–9]. Nitrosocompounds are found in considerable quantities in tobacco smoke, industrial run-off, food products, and certain alcoholic drinks and can be synthesized endogenously under the acidic conditions of the stomach. Because of the practical importance of nitrosocompounds, especially in light of widespread smoking and ethanol consumption, biochemists and oncologists have shown growing interest in nitrosamines and nitrosamine-related mechanisms of tumor development and how they depend on metabolism. In numerous studies, carried out mostly at the end of the previous century, ethanol was shown to induce activity of microsomal enzymes metabolizing nitrosamines although its effects on their carcinogenic activity appeared to be equivocal: in some cases, it enhanced, whereas in other cases, it attenuated carcinogenesis [10–13]. We believe that these discrepancies may be explained by numerous physiological effects of ethanol consumption [14, 15] and by disregarding the peculiar properties of CYP2E1 during planning of experiments and their execution in vivo. In the majority of the experiments, ethanol and nitrosamines were administered chronically at arbitrary intervals. One should keep in mind that ethanol is not only the inducer of CYP2E1 (the mechanism of induction is stabilization of the enzyme molecules [16, 17]) but also its substrate competitively inhibiting nitrosamine metabolism. Because ethanol is usually administered in the amount 1–2 orders of magnitude greater than the amount of the carcinogen, metabolic processing of the latter starts only after excretion of the bulk of ethanol. After the excretion of ethanol, however, the enzyme undergoes deinduction, and its activity rapidly returns to the normal level [16, 17]. The period

[1] The article was translated by the authors.
*Abbreviations:* DENA, diethylnitrosamine; DMNA, dimethylnitrosamine; CYP2E1, cytochrome P450 2E1.

when ethanol ceases to act as the inhibitor of nitrosamine metabolism—and when the ethanol-induced activity of CYP2E1 is still present—is short (less than a day [18, 19]). Therefore, during chronic administration of a carcinogen (a prerequisite of tumor induction in the liver of adult animals), it is usually difficult to decide whether ethanol acts as an inducer or as an inhibitor of carcinogen metabolism in a given case. There are indications in the literature that isopropanol is a more potent inducer of CYP2E1 than ethanol is [18] and that fasting significantly enhances the effects of inducers [20]. Therefore, in this study, we induced this enzyme in mice by a single intraperitoneal (i.p.) injection of isopropanol and by brief fasting (24 h). In our view, it is correct to use suckling (12- to 14-day-old) mice to elucidate how the metabolism of DENA affects its carcinogenic action because a single dose of a carcinogen is sufficient for induction of hepatic tumors in these mice, and therefore, it is possible to distinguish the stimulatory effect of an alcohol on the enzyme and competitive inhibition by the alcohol of the xenobiotic's metabolism. In these experimental settings, it is possible to study the influence of DENA's metabolism on its carcinogenic action by injecting the nitrosamine sometime after the injection of the alcohol, when the latter is already excreted but the alcohol-induced Cyp2e1 activity is still present. Recently, Kang and coauthors [4] tried to clarify this issue in a different way, namely, they tested whether single administration of DENA during the suckling period would induce tumors in genetically modified (Cyp2e1 knockout) mice. In accordance with the prevailing paradigm, the authors expected that no tumors would develop in the experimental animals.

In contrast, such mice demonstrated a delay rather than abrogation of hepatic tumor development. In the present study, we attempted to elucidate the influence of stimulation of DENA metabolism on DENA's carcinogenic effect. We hypothesized that an increase in Cyp2e1 activity at the moment of DENA administration to suckling mice should either enhance the carcinogenic effect (if DENA metabolites are the active agents) or attenuate this effect (if the parent compound [DENA] is the active agent). Our results show that the stimulation of DENA metabolism enhances its hepatotoxic effect but attenuates the general toxicity and hepatocarcinogenic action.

## MATERIALS AND METHODS

**The animals.** Mice of the ICR strain that we used throughout the experiments were obtained from the animal facility of the Institute of Cytology and Genetics, the Siberian Branch of the Russian Academy of Sciences. They were housed in plastic boxes under the conditions of natural lighting and received pelleted food (Chara, Sergiev Posad, Russia) and water **ad libitum.** All procedures and experiments with animals in this study were in compliance with the European Communities Council Directive (86/1986/EEC).

**Analysis of deethylation of DENA.** To study the conditions for stimulation of DENA metabolism in suckling mice, we separated the pups from the mothers and induced Cyp2e1 activity by injecting the pups with a 20% isopropanol solution (100 μL per 10 g of body weight) and by subjecting them to 24-h fasting. After that, the mice were decapitated, and their pooled livers were homogenized in 3 volumes of a cold buffer (1.15% KCl, 20 mM Tris-HCl, pH 7.4). Hepatic microsomes and the 9S fraction (is precipitated at 9000 *g*) were isolated from the homogenates by differential centrifugation. The protein concentration was determined by means of the Coomassie dye G 250 with bovine serum albumin as a standard. Cyp2e1 activity was measured using its specific substrate p-nitrophenol as described previously [21] and DENA (Serva, Germany), whose deethylation was quantified by the method of Chau et al. [22] with modifications of Lee et al. [23]. Microsomes from the livers of untreated age-matched suckling mice served as a control.

**Induction and assessment of carcinogenesis in the liver.** In the hepatocarcinogenesis experiments, we used suckling male mice half of which at age 12 days were injected with isopropanol as described above and were separated from the mothers. The remaining (control) pups were left without treatment, with the mothers. Twenty-four hours after isopropanol administration, both experimental and control mice received DENA i.p. at 50 μg/(g body weight) and were returned to the original mothers; we took away female pups from these mothers. After another maternal separation at the age of one month, the control and experimental mice were kept together throughout the experiment under standard conditions in groups of 6–7 individuals per cage (36 × 20 × 15 cm). At the age of 8.5 months, during 3–5 days, the animals were fed with ground feed containing 3% ferrous sulfate, after which they were decapitated. The livers were weighed, divided into lobes, and incubated for 2–3 h in a mixture of equal volumes of 30% formalin, 2 N hydrochloric acid, and 5% potassium ferrocyanide to detect iron ions [24, 25]. The tumors and pretumorous neoplasms were identified as iron-deficient nodules and measured using an ocular micrometer under a binocular lens (×10 magnification).

**Toxicological experiments.** The general toxicity and hepatotoxic activity of DENA was studied on suckling and adult male mice. A loss of body weight and death were used as general toxicity indicators, and the serum activity of serum alanine aminotransferase served as a measure of hepatotoxicity. To differentiate the toxic action of DENA metabolites and that of the parent compound, the latter was administered to the animals either after Cyp2e1 induction by isopropanol and fasting as described above or as a mixture with iso-

**Table 1.** The influence of isopropanol and 1-day fasting on the activity of the enzymes metabolizing diethylnitrosamine in the liver of suckling mice of the ICR strain

| Enzymatic activity | No. of experiments | Control | Induction by isopropanol + fasting | Factor of induction |
|---|---|---|---|---|
| *p*-Nitrophenol hydroxylase, nmol *p*-nitrocatechol/(min mg protein) | 4 | 1.96 ± 0.31 | 4.78 ± 0.97* | 2.44 |
| N-Diethylnitrosamine deethylase, nmol acetaldehyde/(min mg protein) | 2 | 2.19<br>0.91 | 5.42<br>2.40 | 2.47<br>2.64 |

The number of individual assays performed on pooled microsomes from a group of 4 pups. The data are expressed as $M \pm m$. *—Significantly different from the control, $p < 0.05$.

propanol (2 g/[kg body weight]). The mixture was administered i.p. in the volume of 1 mL per 100 g of body weight. The initial body weight and its change during the experiment relative to the initial body weight were recorded. To assess the hepatotoxic effect, the experimental and control animals were decapitated; the activity of serum alanine aminotransferase was measured using a Vector-Best kit (Novosibirsk, Russia) and expressed in micromoles of the product per milliliter of serum per hour.

**The SOS-chromotest.** The SOS-chromotest on *E. coli* strain PQ37 was used to assess genotoxicity. In this strain, the β-galactosidase structural gene *lacZ* is under control of the promoter of din-inducible gene *sfiA*, so that β-galactosidase activity depends on the expression of *sfiA* and is induced during treatment of bacterial cells with various genotoxic compounds. The assay was conducted by the standard method as described previously [26, 27]. To this end, 0.1 mL of overnight culture of the *E. coli* strain was diluted with 5 mL of the LA medium and incubated on a shaker at 37°C for 2–3 h to achieve the concentration of $2 \times 10^8$ cells/mL. Then, 1 mL of the culture was added to 9 mL of a freshly prepared medium (for analysis without metabolic activation, series X) and to 9 mL of the activating medium consisting of 0.2 M Tris (pH 7.4), 330 mM KCl, 80 mM $MgCl_2$, 50 mM glucose-6-phosphate, 150 mM NADP, and 1 mL of the 9S fraction from the mouse livers (to analyze metabolic activation, series Y). We transferred 0.6 mL from each series to glass tubes (with caps) containing 10 μL of either water or a DENA solution and incubated them with shaking for 2 h at 37°C. After the incubation, 30 μL from each tube was transferred to two new tubes, one of which was used to determine the activity of β-galactosidase, and the other the activity of alkaline phosphatase. To lyse the cells, 270 μL of 0.2 M sodium phosphate buffer, pH 7.75, containing 0.1 M KCl, 10 mM $MgSO_4$, 0.3 mM dithiothreitol, and 0.1% SDS, was added into each tube, and the tubes were incubated in a thermostat for 10–15 min at 37°C. The β-galactosidase reaction was initiated by the addition of 60 μL of an *ortho*-nitrophenyl-β-galactoside solution (4 mg/mL) and was stopped in 30 min by addition of 200 μL of 1 M $Na_2CO_3$. Absorbance of the solutions was measured at 405 nm against a cell-free control. The activity of alkaline phosphatase was measured using the buffer consisting of 1 M Tris-HCl pH 8.05, 0.1% SDS, and *p*-nitrophenylphosphate (4 mg/mL) as a substrate; the reaction was stopped by addition of 200 μL of 1.5 M NaOH. The effect of treatment on viability of the bacteria was assessed by the ratio of alkaline phosphatase activity in the experiment to that in the control, whereas genotoxicity was determined as the factor of induction (FI) by means of the formula $FI = R_o/R_c$, where $R_o$ is the ratio of β-galactosidase to alkaline phosphatase activity in the experiment, whereas $R_c$ is the same ratio in the control (without DENA).

**Statistics.** The results were analyzed statistically using Microsoft Excel 7.0; the significance of differences between the means was determined by Student's *t* test.

## RESULTS AND DISCUSSION

**Induction of cytochrome P450 2e1.** As shown in Table 1, the metabolizing activity of microsomes toward *para*-nitrophenol was 2.5-fold greater in isopropanol-treated fasting suckling mice than in the control ($p < 0.05$). Direct measurement of the DENA metabolism rate by acetaldehyde formation also yielded a 2.5-fold increase in this rate in the pretreated animals as compared to the control (Table 1). These findings indicated that our relatively nontoxic method produces a sufficiently high level of Cyp2e1 induction in the liver and can be used for elucidation of the role of DENA metabolism in its hepatocarcinogenic effect.

**Metabolic processing diminishes the carcinogenic effect of DENA.** Table 2 shows that hepatic tumor nodules developed in 100% of animals both in the control and experimental groups; however, in the experimental group, the average number of the nodules was 1.8-fold smaller, and the average number of large nodules (at least 2 mm in diameter) was almost 5-fold smaller than in the control ($p < 0.001$). These results were also confirmed in another experiment on seventeen slightly older (15- to 16-day-old) mice, in which, on average, DENA induced the development

**Table 2.** Incidence and multiplicity of hepatic neoplastic lesions in male ICR mice after i.p. administration of diethylnitrosamine (DENA): either 24 h after i.p. injection of isopropanol and fasting or no other treatment (control)

| | Control, DENA only | Experiment, isopropanol + fasting + DENA |
|---|---|---|
| Total number of mice | 18 | 20 |
| Percentage of mice with hepatic nodules, % | 18 (100) | 20 (100) |
| Lifespan, days | 259.0 ± 4.1 | 253.0 ± 3.3 |
| Body weight, g | 36.7 ± 1.2 | 38.6 ± 0.9 |
| Relative liver weight, % | 5.89 ± 0.34 | 4.84 ± 0.18* |
| Total number of hepatic nodules | 79.0 ± 7.3 | 43.0 ± 6.2** |
| Nodules at least 2 mm in diameter | 19.0 ± 4.0 | 4.0 ± 1.1** |
| Mean diameter of largest hepatic nodule, mm | 5.2 ± 0.61 | 2.50 ± 0.27** |

Isopropanol (as a 20% solution) was injected i.p. at 0.1 mL per 10 g of body weight at age 12 days and the animals were deprived of food for 24 h. DENA was administered i.p. at 50 μg/g body weight at age 13 days. The tumorous lesions in the liver were identified as iron-deficient nodules after Perls staining [25]. The data are expressed as $M \pm m$. The asterisks indicate significant differences from the control; ** $p < 0.01$; *** $p < 0.001$.

of 34 ± 5.5 tumor nodules in the control pups and only 14 ± 3.9 nodules in the isopropanol-treated mice that fasted before the DENA administration. The doses and method of treatment were the same as those in the previous experiment; therefore, the number of tumors was apparently affected by the older age of the mice at the time of treatment. Nevertheless, in this case, the difference between the experiment and control was also significant ($p < 0.01$).

**Inhibition of metabolism diminishes hepatotoxicity but enhances general toxicity of DENA.** In order to confirm that the decrease in the carcinogenic effect of DENA in our experiments is actually caused by the stimulation of its metabolism, we tried the opposite approach, i.e., inhibition of DENA metabolism. For this purpose, we used the competitive inhibitor of DENA metabolism (isopropanol), which was administered to 12-day-old mice as a mixture with DENA. The doses of isopropanol (2 g/kg) and DENA (50 mg/kg) were similar to those used in the previous experiments where these chemicals were tolerated well by the animals when administered separately. The combined administration killed all mice on average 120 ± 15.7 h after the treatment; this situation did not allow us to analyze the effects of inhibition of DENA metabolism on its carcinogenic effect. At the time, we were unable to repeat the experiments at lower DENA doses with all necessary control groups of mice. Therefore, to study the toxic effects of the combined administration of isopropanol and DENA, several experiments were performed on adult mice. Toxicity was assessed as damage to liver cells; the latter effect is usually measured as an increase in serum alanine aminotransferase activity. At the same dose of isopropanol (2 g/kg), the dose of DENA was increased to 150 mg/kg. The results are presented in Table 3. Unexpectedly, isopropanol when administered 1 day before DENA was found to increase, but when administered simultaneously with DENA, to decrease its injurious action on the liver. By itself (without DENA), isopropanol had no effect on the activity of alanine aminotransferase in mouse blood.

To compare the results with those obtained in the suckling mice, in the subsequent series of experiments on adult mice, we used a loss of body weight as an indicator of general toxicity. As shown in the figure, after DENA administration (200 mg/kg in this experiment), the body weight of mice decreased linearly during 4 days, after which it stabilized at the level of 80% of the initial weight and subsequently increased slowly (curve *3*). After the combined administration of DENA and isopropanol (curve *4*), during four days, the body weight declined to less than 75% of the initial value, and during the following 4 days, all the animals died. In contrast, the administration of isopropanol and fasting for 24 h had a significant protective effect against the toxic action of the subsequently administered DENA (curve *2*). Without DENA administration, the isopropanol and fasting caused a ~10% decrease in body weight, which returned to normal within 3 days after resumption of feeding (curve *1*).

Thus, the hepatotoxicity of DENA does not correlate with its general toxicity and hepatocarcinogenicity. Hepatocytes are damaged probably by alkylating metabolic products of DENA, whereas general toxicity (assessed as body weight loss and deaths) and hepatocarcinogenicity are caused primarily by the parent compound.

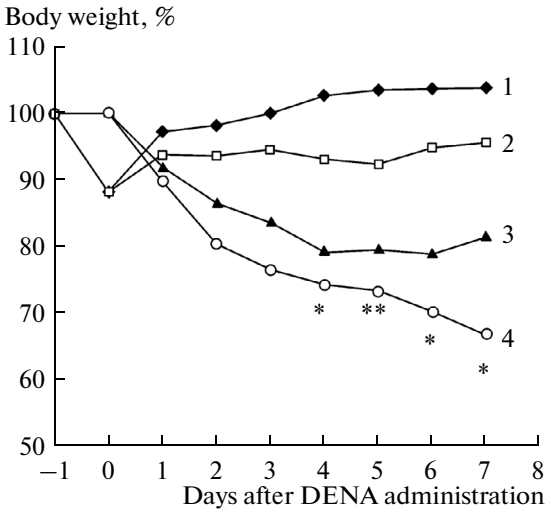
We recently reported direct toxic effects of unmetabolized DENA on *S. typhimurium* in an Ames test [27]. A similar effect was uncovered earlier in dogs: inhalation of dimethylnitrosamine fumes caused acute lethality with insignificant hepatic damage [1]. It is still unclear what causes this general toxicity of DENA and whether it is a consequence of organ damage or a regulatory aberration. It should be noted, however,

**Table 3.** The effects of isopropanol as an inducer of Cyp2e1 activity in the liver (isopropanol + fasting) and as the inhibitor of DENA metabolism (when mixed with DENA) on hepatotoxic activity of DENA in mice

| Group and effect | Activity of alanine aminotransferase in blood (μM/(mL h)) after DENA administration | |
|---|---|---|
| | In 24 h | In 48 h |
| 1. DENA on day 0 | 12.0 ± 1.1 (6) | 35.0 ± 8.0** (3) |
| 2. Isopropanol + fasting one day before DENA injection, DENA on day 0 | 29.0 ± 7.5* (6) | 64.0 ± 12.2** (3) |
| 3. Isopropanol + DENA as a mixture on day 0 | 7.0 ± 2.0 (7) | 7.7 ± 3.7 (3) |
| 4. Isopropanol one day before DENA injection | – | 5.4 ± 0.9 (3) |
| 5. Control, no treatment | – | 5.3 ± 0.7 (4) |

Male ICR mice at age 2.5 months were used in the experiments. Isopropanol (2 mL/kg as a 20% solution) was injected i.p., and the animals were deprived of food for 24 h. DENA was administered i.p. at 150 mg/kg. The mice were decapitated 24 or 48 h after DENA administration or 48 or 72 h after isopropanol administration. The number of animals is indicated in brackets. The data are expressed as $M \pm m$. *—Significantly different from groups 1 and 3 ($p < 0.05$); **—significantly different from groups 3, 4, and 5 ($p < 0.05$–$0.01$).

that this general toxicity is positively related to the hepatocarcinogenic action of DENA on the liver. One article [28] lists numerous examples when the carcinogenic action of nitrosamines does not coincide with their DNA-damaging effects, i.e., the carcinogenic effect is caused by the unactivated compound (without the involvement of alkylating metabolites). The mechanism via which unmetabolized (nonmutagenic) compounds may initiate tumor development were outlined briefly elsewhere [29–33] and will not be discussed here. Here, it seems relevant to mention the results of our SOS-chromotest.




DENA was administered i.p. on day 0 at 200 mg/kg. To induce Cyp2e1 activity, on day –1, the mice were injected i.p. with isopropanol at 2 mL/kg and deprived of food for 24 h (Group 1). Group 2: induction of Cyp2e1 as in Group 1 plus DENA on day 0. Group 3: DENA only on day 0. Group 4: DENA in a mixture with isopropanol i.p. at the aforementioned doses on day 0. The X-axis shows time points (days) relative to DENA administration; the Y-axis: body weight as a percentage of the initial body weight (1 day before DENA injection). Four animals per group in Groups 1–3, five animals in Group 4. The time points of death are marked by asterisks.

**SOS-chromotest.** Table 4 shows that DENA dose-dependently reduces viability of *E. coli* and has genotoxic effects, but addition of the 9S fraction from mouse livers to the incubation medium protected the bacteria from both the toxic and "mutagenic" effects of DENA. The quotation marks are used because in this test, the mutagenicity is evaluated by activity of the enzymes co expressed with certain genes involved in the repair of bacterial DNA in response to the damage caused by disruption of DNA replication. On the other hand, the genotoxic effect can be mimicked by the substances activating these enzymes without the involvement of DNA damage. This is possible, for example, for the substances acting directly on the enzyme molecules or on intermediate mechanisms underlying the transmission of information about DNA damage to DNA repair systems. In this case, the effect can be achieved not only through the chemical interaction but also through a physicochemical interaction of the chemical agent with the protein molecule, as we showed previously for tempol, a substance stimulating the activity of aldehyde dehydrogenase in the mouse liver [34]. Furthermore, DENA may have an effect on the expression of some genes by influencing the activity of the transcription factors, as is the case for hepatic cells of mice and rats [35, 36]. Moreover, one study [37] shows that 6 h after DENA administration, strong expression of the Oct4 transcription factor is observed in mouse hepatocytes. This transcription factor is normally expressed in embryonic cells but not in differentiated hepatic cells. We believe that this and other similar effects of DENA are linked to its carcinogenic properties.

As mentioned in the Introduction section, Kang et al. [4] observed slowing but not abrogation of DENA-induced formation of liver tumors in *Cyp2e1* knockout mice. According to the traditional understanding of tumor initiation by genotoxic metabolites

**Table 4.** General toxic and DNA-damaging effects of DENA in a *E. coli* PQ37 genotoxicity assay (SOS-chromotest) in the absence (above the line) or presence (below the line) of the cytosol and microsomes (9S fraction) from the mouse livers

| Sample | Dose of DENA (μg/sample) | Viable bacteria (%) | Factor of induction |
|---|---|---|---|
| 1 | 0.19 | 67<br>95 | 1.99<br>1.18 |
| 2 | 0.24 | 63<br>78 | 1.70<br>1.44 |
| 3 | 0.24 | 57<br>73 | 2.42<br>0.92 |
| 4–8 | 0.32 | $47.2 \pm 2.0$<br>$71.2 \pm 2.50$* | $3.30 \pm 0.24$<br>$1.42 \pm 0.07$* |

The analytical method is described in Materials and Methods. The 9S fraction was obtained by centrifugation of 25% liver homogenates from suckling ICR mice. The factor of induction (FI) was determined by means of the formula $FI = R_o/R_c$, where $R_o$ is the ratio of β-galactosidase activity to alkaline phosphatase activity in the experiment, and $R_c$ is this ratio in the control (without DENA). 3,4-Benzopyrene (BP, 2.5 μg per assay) served as a positive control. Under our experimental conditions, FI for BP was $3.0 \pm 0.2$ (1.0 without the 9S fraction). * A significant difference from the data above the line, $p < 0.001$.

of DENA, Kang et al. stated that the metabolic activation of DENA was probably performed by other cytochrome P450 isoforms, in particular 2A6. Nonetheless, the studies [38] and [39] that are cited by Kang et al. [4] involve adult mice and human cytochromes. Kang et al. [4] administered DENA to 14-day-old mice, whereas *Cyp2a5*, the mouse analog of human CYP2A6, starts to be expressed in the liver of these mice only by the end of the third week of life [42].

In conclusion, it is necessary to discuss the possible reason for the seeming discrepancy between the results obtained in our experiments (attenuation of the carcinogenic effect of DENA during stimulation of its metabolism) and the results of Kang et al. [4] (weakening of the carcinogenic effects of DENA during inhibition of its metabolism: in *Cyp2e1* knockout mice). In our experiments, the differences between the experimental and control groups of mice were detectable at the stage of carcinogenesis initiation, namely during the first hours or days after DENA administration, when there were differences in *Cyp2e1* activity in the liver.

Afterwards, until the end of the experiment, the enzymatic status of the experimental and control animals was the same; this situation rules out the differences in tumor progression. By contrast, in the experiments of Kang et al. [4], the knockout mice differed from the control mice not only at the initiation stage but also afterwards, at the stages of carcinogenesis promotion and tumor progression. In healthy animals, CYP2E1—while metabolizing endogenic substrates—causes oxidative stress and provokes an inflammatory reaction among nonparenchymatous hepatic cells; the latter then secrete cytokines and growth factors that stimulate compensatory proliferation of initiated hepatocytes and promote the development of preneoplastic nodules and tumors [41–43]. These phenomena do not take place in *Cyp2e1* knockout mice. This means that tumor initiation in suckling mice without DENA metabolism—not the decelerated development of hepatic tumors (versus control)—should be taken into account when interpreting the results of Kang et al. [4]. Thus, the latter data are consistent with (not contradictory to) our findings.

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