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THE *IN OVO* CARCINOGENICITY ASSAY (IOCA): A REVIEW OF AN EXPERIMENTAL APPROACH FOR RESEARCH ON CARCINOGENESIS AND CARCINOGENICITY TESTING

Harald Enzmann[1] and Klaus D. Brunnemann[2]

[1]Bayer AG, Institute of Toxicology, 42096 Wuppertal, Germany and [2]American Health Foundation, Valhalla, NY 10595, USA

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1. ABSTRACT:

Up to now, the carcinogenicity of a substance, i.e., its cancer-causing effect, has been usually determined with the help of extensive animal testing. The *in ovo* carcinogenicity assay (IOCA) has been suggested as a rapid and inexpensive, non-animal, method for carcinogenicity testing and for experimental studies on mechanisms of carcinogenesis. The substance to be tested is injected into a fertilized ovum. No later than four days before the hatching date, the embryos are released, and the liver is removed for identifying the effect of hepatocarcinogens.

Histological, cytological and molecular biological alterations of the embryonic liver have been induced with a variety of chemical carcinogens. After sufficiently high doses of hepatocarcinogens, tubular structures predominate in the liver and replace the normal trabecular pattern. The cell and nuclear size of the hepatocytes in embryonic liver is severely increased after exposure to chemical carcinogens over a wide dose range including doses that fail to elicit cytotoxicity in the embryonic liver.

The *in ovo* exposure of avian embryos to hepatocarcinogens has also been shown to induced focal lesions in the embryonic livers. In embryonic turkey liver, *in ovo* exposure to carcinogens mostly induced basophilic hepatocellular altered foci (foci of altered hepatocytes) that were similar to carcinogen-induced lesions in rodent liver. In embryonic quail livers, most of the focal lesions were composed of cells that morphologically appeared more similar to cholangiocytes than to hepatocytes.

The *in ovo* exposure of embryonic liver to hepatocarcinogens resulted in damage to the mitochondrial DNA (mtDNA). Electrophoresis on agarose gels revealed a carcinogen-induced effect on the molecular size of the mtDNA. The content of mtDNA of the regular size of 16 kilobases (kb) dose-dependently decreased. At the same time, the amount of fragmented mtDNA of various sizes increased.

The advantages of bird embryos for carcinogenicity testing are considerable: The IOCA is rapid, since the *in ovo* phase does not exceed 24 days. Since no facilities for animal housing are required, it is less expensive than whole animal experiments. There is less potential of human exposure during the experiment than in animal experiments and studies in a non-rodent system may help to discriminate between chemicals that are rodent specific carcinogens and chemicals that may constitute a potential cancer hazard to man.

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Send correspondence to: Klaus D. Brunnemann: American Health Foundation, 1 Dana Road, Vallhalla, NY 10595 Tel: (914) 789-7167, Fax: (914) 592-6317, E-mail: nrivera2@ix.netcom.com

Table 1. Nuclear enlargment in % of control value

		CONTROL	NNK 0.3 mg	NNK 1mg	NNK 2mg	NNK 6mg
95% quantile	arith. mean	100.0	102.0	190.8	247.0	318.9
95% quantile	SD	6.4	5.3	14.4	35.4	23.6
95% quantile	arith. mean	100.0	111.2	223.4	282.5	364.9
95% quantile	SD	7.6	6.7	25.1	29.9	32.2

Abbreviations: NNK: 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, arith.mean: arithmetic mean, SD: standard deviation

Table 2. Separate evaluation of periportal and perivenular hepatocyte nuclei

		VALUES OF INDIVIDUAL SAMPLES AREA IN μM^2					GROUP VALUES	
	Sample no.	1	2	3	4	5	Arith.mean	S.D.
Periportal hepatocytes	arith. mean	15.90	17.89	18.86	19.69	17.74	18.02	1.27
	95 percentile	22.91	24.90	25.41	27.51	23.19	24.78	1.66
	99 percentile	29.54	29.14	36.19	46.63	35.56	35.41	6.33
Perivenous hepatocytes	arith. mean	16.75	16.24	19.07	18.95	116.91	17.59	1.19
	95 percentile	22.03	20.64	26.56	30.75	22.08	24.41	3.75
	99 percentile	23.47	23.24	41.27	41.90	23.87	30.75	8.85

Abbreviations: arith.mean: arithmetic mean,SD: standard deviation

2. INTRODUCTION: AVIAN MODELS OF CARCINOGENICITY

The use of birds in experimental cancer research is even older than the experimental induction of tumors by chemicals. Four years before the first chemical induction of an experimental tumor was described in rabbits, the viral induction of chicken sarcomas was published. Since then, the experimental induction of carcinogenesis in birds has been predominantly used for studies on viral carcinogenesis. The sensitivity of birds to chemical carcinogens became obvious with the elucidation of the turkey X disease which resulted in the discovery of the strongest known carcinogens, the aflatoxins.

More recently, liver cell cancer in birds has emerged as a potential model for the synergistic effects between viral hepatitis and exposure to chemical carcinogens Bird embryos have been known to be more sensitive to viral transformation than the hatched birds. The use of bird embryos has also been suggested for tests for genotoxicity demonstrating chemically induced damage to nuclear DNA.

The disadvantage of this experimental approach are the potential metabolic differences between this non-mammalian system and the rodents usually used for carcinogenicity studies. Depending on the test chemical used, activation or inactivation of the administered chemicals in the embryonic tissue may be a crucial determinant. Drug-metabolizing enzyme activities in turkey and chick liver have been shown to be similar to the activities in rat liver. In the embryonic liver, however, the activation of xenobiotics is less effective than in adult liver or at hatching. Nevertheless, measurable enzyme activities are found as early as on day 5 of development. Since the mixed function oxidase enzyme system can be induced even at this early stage, the sequential administration of an enzyme inducing agent and of the potentially carcinogenic test substance might be advantageous.

3. CARCINOGEN-INDUCED CELLULAR CHANGES IN EMBRYONIC AVIAN LIVER

3.1 Induction of adenoid formations by carcinogens

Exposure of avian embryonic liver to the hepatocarcinogenic nitrosamines N-nitrosomorpholine (NNM) and diethylnitrosamine (DEN) resulted in the occurrence of tubular (adenoid, ductular) formations instead of the physiological trabecular organization of the embryonic liver (10, 11). Extracellular deposits of a brownish substance (bile pigment) were occasionally

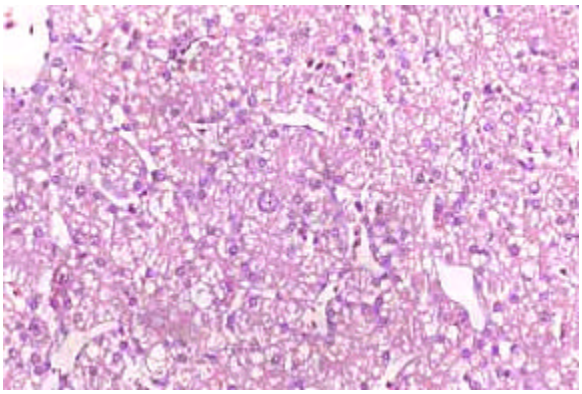

Figure 1: Effect of diethylnitrosamine on nuclear size: obviously enlarged hepatocyte nucleus (center) after exposure to 0.4 mg of diethylnitrosamine. Hematoxylin eosin stain, objective magnification 40-fold.

observed in these cell populations. Neither basal membranes nor fibrillary structures were detectable at the luminar cell pole. After high doses of the nitrosamines, the whole liver was composed of a mixture of small basophilic hepatocytes and large acidophilic hepatocytes including cells with a ground glass appearance. A similar mixture of large acidophilic hepatocytes and small basophilic hepatocytes has long been shown in the liver after exposure of rats to the very same nitrosamines (12). The occurrence of tubular structures is a typical histological feature of adenoid hepatocellular tumors (13), but may also be observed in severely damaged human liver (14,15).

3.2 Carcinogen-induced hepatocellular nuclear enlargement

The cell and nuclear size of the hepatocytes in embryonic liver is severely increased after exposure to chemical carcinogens. This effect on the nuclear size is dose dependent (table 1) and occurs at doses lower than the doses required for the induction of the tubular formations or preneoplastic focal lesions (see below). Single hepatocytes with unusually large nuclei (figure 1) are observed after exposure to low doses that fail to elicit cytotoxicity in the embryonic liver. At low doses, only a small minority of hepatocytes exhibit enlarged nuclei after exposure to low doses of carcinogens whereas the vast majority of nuclei are apparently normal. Therefore, the 95% quantile or the 99% quantile rather than the arithmetic mean or the median of a sample is the most appropriate means of assessing the exposure-induced nuclear size differences.

3.3 Quantification of carcinogen-induced nuclear enlargement

The measurement of cell and nuclear size may be performed using flow cytometry (16). Most hepatotoxins, however, cause qualitatively and quantitatively different damage to periportal and perivenous hepatocytes (17,18) and cell suspensions are not suitable for the discrimination of periportal and perivenous hepatocytes. In addition, cell suspensions do not allow an exact distinction between cells of the carcinogen-induced focal lesions (see below) and the surrounding extrafocal tissue. Therefore, we preferred to measure nuclear size in the tissue sections. There were no differences in nuclear size between periportal and perivenous hepatocytes in untreated control embryos (table 2).

It is worth to consider that thickness of the section may be a crucial point in the planimetric measurement of nuclear areas. The measured areas of peripherally cut nuclei (small areas) strongly depends on the thickness of the section. Focusing the microscope for the maximal nuclear area will have the consequence that with an infinite increase in section thickness no peripherally cut nuclei, but exclusively the maximal (central) area of nuclei is measured. On the other hand, the ideally central profiles will give the maximal area values both in infinitely thin and in infinitely thick sections. Therefore, whereas small profiles may be underrepresented in the measured data and their number may be biased by the thickness of the section, the maximal area values will hardly be biased by variations in thickness of the section, if enough profiles per sample are measured.

3.4 Significance of the carcinogen-induced nuclear enlargement

The size of liver cell nuclei may reflect various physiological and pathological processes. In rodents, distinct alterations in the ploidy pattern are characteristic during postnatal development (19). Alterations of the nuclear size of parenchymal cells have frequently been observed during various stages of hepatocarcinogenesis. Various authors reported the occurrence of enlarged or hyperploid nuclei at early stages of the carcinogenic process (20-24), effects of the carcinogenic treatment on the number of binucleated hepatocytes (24-27) or proposed *in vitro* test systems based on the carcinogen-induced nuclear enlargement (28).

At least some of these carcinogen-induced phenomena seemed also to be linked to liver regeneration. Regeneration after partial hepatectomy (29-31) or after toxic injury (32) results in a shift towards higher ploidy levels and a reduced number of binucleated hepatocytes. Consequently, the occurrence of enlarged nuclei during chemically induced hepatocarcinogenesis was regarded as to be due to the combination of regenerative and toxic effects (33). More recent experiments, however, showed that enlarged nuclei occurred after low doses of carcinogens that failed to induce other signs of nonspecific toxic effects (34). Nuclei with unusually high ploidy level were observed in LEC rats with hereditary hepatitis (35) before these rats developed preneoplastic liver cell foci and hepatocellular carcinomas without any deliberate exposure

Table 3. Comparison of *in ovo* carcinogenesis in turkey and quail parameters of the *in ovo* phase in turkey and quail

	EGG WEIGHT	INCUBATION PERIOD	TERMINATION NOT LATER THAN	MAXIMAL INJECTION VOLUME	POSITIVE CONTROL	REQUIRED INCUBATOR SPACE	REQUIRED AMOUNT OF TEST SUBSTANCE
Turkey	80g	28 days	24 days	1000 µl	1 mg DEN	More	More
Quail	10 g	24 days	21 days	100 µl	100 µg DEN	Less	Less

Abbreviations: DEN: Diethylnitrosamine

Figure 2: Solid basophilic cell focus in embryonic turkey liver after exposure to 1 mg of diethylnitrosamine. Hematoxylin eosin stain, objective magnification 10-fold.

to carcinogens (36). According to Clawson and coworkers an enlargement of nuclei was also observed after, low, and essentially non-toxic doses of mutagenic carcinogens (37). Similarly, effects on the nuclear size were observed in the IOCA even after small doses that did not induce foci of altered hepatocytes and did not induce nonspecific toxic effects, such as cell death, loss of glycogen or fatty change. This observation is not compatible with the assumption, that the enlarged nuclei merely reflect a compensatory regeneration due to toxic cell loss. Both the occurrence of aneuploid or hyperploid nuclei caused by nonspecific effects on the mitotic spindle (38) and a carcinogen-mediated acceleration of the naturally occurring polyploidization program (39) may account for the observed effects.

These findings suggest that the occurrence of enlarged nuclei at early stages of experimental carcinogenesis, induced by low doses of chemical carcinogens indicate cellular changes, that may be involved in the carcinogenic process.

4. CARCINOGEN INDUCED FOCAL HEPATIC LESIONS

4.1 Experimental induction of focal hepatic lesions in rodents

In rodent liver, the induction of altered foci is widely used as a rapid model for hepatocarcinogenesis (40-42). Sasaki and Yoshida (43) were the first to notice that foci of altered hepatocytes, consisting of clear and basophilic cells, precede the occurrence of chemically-induced liver tumors. During the last few years, further subtypes of altered hepatocellular foci showing

characteristic morphological or metabolic alterations were found (44,45). Several morphometric studies support a predominant developmental sequence from clear cell foci through mixed and basophilic cell foci to hepatomas (12,46-50). However, different types of foci not related to this sequence have also been observed (45,51-54). This concept is further supported by the demonstration of a similar developmental sequence of metabolic alterations (55,56) and increasing cell proliferation (57).

Since the occurrence of these foci is perceivable only in tissue not in cell culture systems, experiments in whole animals are required. Nevertheless immortalized cell lines have been described which are phenotypically similar to these foci observed *in situ* (58).

4.2 Induction of focal hepatic lesions in avian embryonic liver

The heterogeneity of focal lesions observed in avian embryonic liver corresponds to a similar heterogeneity in rodent liver (10-12,45,59). Turkey and quail have been preferred over chicken eggs because of the longer incubation period of turkey eggs (28 days) and quail eggs (24 days) as compared with 21 days in chicken. Assuming, that the development of phenotypically altered preneoplastic hepatic foci requires some time, a longer hatching period may be more suitable. The features of these experimental models are outlined in table 3.

In the turkey embryos foci of altered hepatocytes can be demonstrated. They are similar to preneoplastic liver cell foci that have been observed in the liver of rodents after exposure to carcinogens. In HE stained sections, *in ovo* exposure to carcinogens results in the occurrence of mostly basophilic cell foci. Basophilic cell foci were solid (figure 2) or showed an adenoid structure (figure 3). Less frequently, clear cell foci were observed.

Quail eggs have also been used for the exposure of avian embryos to chemical carcinogens (60). Similar to the findings in embryonic turkey liver, high doses of

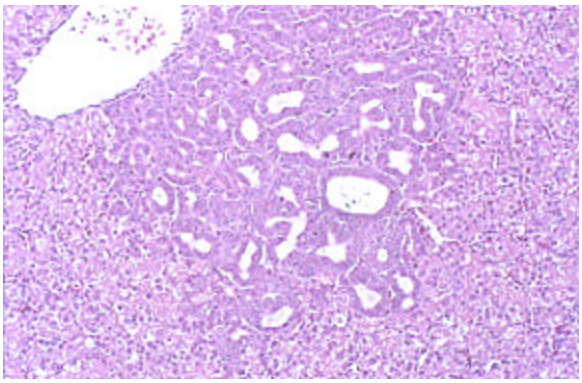

Figure 3: Adenoid basophilic cell focus in embryonic turkey liver after exposure to 2 mg of diethylnitrosamine. Hematoxylin eosin stain, objective magnification 20-fold.

diethylnitrosamine or N-nitrosomorpholine induced foci of altered hepatocytes in embryonic quail liver (61). However, after lower doses of the nitrosamines, the focal lesions in quail were mostly composed of cells, morphologically more similar to cholangiocytes than to hepatocytes. These cells were arranged in distinct glandular patterns.

After high doses of chemical carcinogens, reversibility of focal and nodular hepatocellular lesions rather than progression to fully transformed neoplastic lesions has been described (61-63,64,65). Similar phenomena cannot be exclude for foci induced by high doses of carcinogens that elicit significant liver damage. However, there is no evidence for the reversibility of foci of altered hepatocytes induced by low doses of hepatocarcinogens that do not appreciably alter the surrounding liver (figure 3).

The demonstration of decreased activity of glycogen phosphorylase was the most reliable enzyme histochemical marker *in ovo*. Similar to findings in rodents (56,66), the majority of the foci of altered hepatocytes showed a decreased activity of glycogen phosphorylase. In addition some foci showed a more violet tinged staining of the reaction product. A similar phenomenon was described in aged control rats (45) and in rats fed a choline-deficient diet. N-nitrosomorpholine induced glycogen storage and increased glucose-6-phosphate dehydrogenase activity in foci of altered hepatocytes in a dose dependent manner. However there was no increase in foci with elevated activity of glycogen phosphorylase (68).

4.3 Focal hepatic lesions as endpoints in carcinogenicity testing

In rodents, foci of altered hepatocytes are regarded as preneoplastic lesions. These lesions may progress to benign and malignant liver cell tumors without any additional exposure. Therefore in rodents foci of altered hepatocytes have frequently been used as end points in carcinogenicity testing (41,42). Testing based on the induction of tumors in whole animals usually requires more than two years. Using preneoplastic lesions as end points in carcinogenicity testing may shorten the required time to a few months. Since these experiments are terminated before tumors occur, the animals do not suffer from tumor growth or metastasis. However, some frequently used protocols include partial hepatectomy for the enhancement of the carcinogenic effect by regenerative proliferation, thus requiring abdominal surgery in animals already exposed to the test chemical (75).

Since foci of altered hepatocytes induced in animal experiments have been successfully used as endpoints in carcinogenicity testing, the occurrence of similar lesions in the IOCA may offer an even more accelerated and convenient approach to carcinogenicity testing. It is generally believed that the long term experiments in carcinogenicity testing are necessary for a sufficiently high sensitivity. However, at least for the detection of the carcinogenic effects of small doses of potent carcinogens, the IOCA based on the induction of focal liver lesions seems to be as sensitive as the chronic rodent bioassay which is based on the occurrence of tumors.

5. INDUCTION OF MTDNA DAMAGE *IN OVO*

5.1 mtDNA as target for genotoxic chemicals

It is becoming clear that alterations of mitochondrial DNA (mtDNA) may be an alternative and perhaps a superior indicator for genotoxic effects (76). DNA damage may be the result of a direct reaction between a genotoxic chemical and the target DNA molecule. It may also be mediated by free radicals (77). Consequently, mtDNA seems more likely to be a target for the effect of these chemicals than the nuclear DNA (nDNA). In addition, several differences between nDNA and mtDNA may contribute to the higher sensitivity of mtDNA to genotoxic effects. This may, to some extent be due to the close association of nDNA with histone and non-histone proteins that may offer some protection, whereas the mtDNA appears much more exposed to genotoxic effects (78). Damage to nuclear DNA may be efficiently removed by repair systems, but mtDNA has a significantly lower DNA repair capacity (79). Furthermore, the D-loop structure of mtDNA is unique insofar as it contains a single-stranded DNA structure which controls both transcription and replication (80). Since single- stranded DNA is more easily damaged by various genotoxic mechanisms, the D-loop may be a natural hot spot for experimentally induced DNA damage (81). In addition, damage to multiple sites on nDNA may, to some extent, be self-limiting, since the enzymes for DNA replication are coded by nDNA. Severe damage to these genes may result in inactivation of enzymes and the inability of damaged cells to replicate. A similar mechanism may not occur after damage to the mtDNA since the enzymes required for the replication of mtDNA

Table 4. Effect of different doses of NNM on mtDNA SBQ (in % of control)

	NATIVE		RNase	
Dose	A.M.	S.D.	A.M.	S.D.
Control	100	8	100	5
2 mg	115	13	114	9
4 mg	148	34	176	54
8 mg	226	52	296	95

Abbreviations: NNM: N-nitrosomorpholine; mtDNA: mitochondrial DNA; SBQ: smear-band-quotient; A.M.: arthmetic mean; S.D.: standard deviation. The SBQ gives the ratio of mtDNA fragments to intact mtDNA of regular size.

are coded in the nuclear genome (78). In contrast, mutated mitochondria may accumulate in cells and eventually dominate in certain daughter cells of a dividing tissue due to the non-mendelian inheritance of mtDNA (82,83). Another advantage of examining the effect of genotoxic chemicals on mtDNA is its invariability from 16 kb. The supercoiled, relaxed or linearized forms of mtDNA are all detectable by gel electrophoresis (84). Presence of DNA fragments of different sizes most likely reflect the induction of DNA damage. In contrast, during preparation the nDNA (one DNA molecule per chromosome!) usually break, unless very sophisticated methods are used. Consequently, the molecular size of nDNA observed depends on the isolation procedure rather than being reflective of DNA damage.

5.2 Studies of mtDNA using carcinogen-exposed embryonic turkey liver

A disadvantage of mtDNA is that only 0.1% to 1% of total cellular DNA is mtDNA. If methods established for investigations on nuclear DNA are to be used, DNA of about 100 to 1000 fold greater samples are required for experiments with mtDNA as compared with experiments with nuclear DNA. Tissue samples from the IOCA offer a sample size much greater than samples from cell culture systems. The purification of mtDNA from embryonic turkey liver according to the protocol of Welter and coworkers (85) gave mtDNA yields of about 0.2 to 0.4 ng per mg tissue wet weight or up to 0.5 µg mtDNA per liver. The physiologically supercoiled structure of mtDNA was well preserved during the isolation procedure and formed a distinct band in gel electrophoresis. Only small amounts of the mtDNA were of the relaxed form (86).
Separation of native as well as ribonuclease treated samples of mtDNA by gel electrophoresis showed a discrete smear starting at the band of the relaxed form of mtDNA (86).

The electrophoretic separation of mtDNA from turkey embryos previously exposed to hepatocarcinogenic nitrosamines revealed smaller size fragments that could be quantitated by densitometric measurement as smear-band-quotient (SBQ). In experiments with diethylnitrosamine, clear-cut damage to mtDNA was induced over a dose range from 1.24 to 12.4 mmol/kg (86) similar to the concentration range in other *in vitro* tests for genotoxicity (87,88). Table 4 illustrates the effect of the hepatocarcinogenic nitrosamine N-nitrosomorpholine (NNM) on mtDNA in embryonic turkey liver.

6. PERSPECTIVE OF THE *IN OVO* MODEL OF CARCINOGENESIS

In ovo experiments may fill the gap between experiments with whole animals and cell culture systems, combining some advantages of both approaches. These advantages are summarized below:

1. The IOCA is rapid, since focal and nodular preneoplastic liver lesions can be induced in 24 days and damage to mitochondrial DNA in 4 days.
2. The IOCA is less expensive than whole animal experiments. Since the experiments are terminated several days before hatching and fertilized eggs are commercially available, facilities for animal housing are not required.
3. The IOCA is logistically simple: It does not require highly sophisticated methods or expensive equipment since routine histology is sufficient for the detection of the induced nuclear alterations and focal hepatic lesions.
4. Quantification of the induced effects can be easily achieved by simple morphometric methods both for the nuclear enlargement and for the occurrence of focal hepatic lesions.
5. Proliferation-mediated effects on carcinogenesis are unlikely to affect the induction of foci in the IOCA. Similar to the partial hepatectomy in the Ito-model, the rapid growth of the embryonic liver provides such a high cell proliferation that a stimulating effect of the test substance is of little or no effect. Therefore, merely cytotoxic effects are less likely to mimic carcinogenicity.
6. The pronounced proliferation of the embryonic tissue also contributes to the high sensitivity of the assay. The induction of regenerative proliferation in potential target tissues frequently introduced in animal experiments (36, 39) is not necessary *in ovo* due to the high proliferation rate of the embryonic tissue.
7. An additional non-rodent species strengthens the extrapolation of animal studies to humans.
8. Finally, there is less potential of human exposure during the experiment: There is no excretion of the injected carcinogens from the eggs. The egg shell may be regarded as a barrier, at least, for the non-volatile chemicals. The amounts of the carcinogenic chemicals that are needed for the IOCA are relatively small as compared to animal experiments.

It may be possible to study carcinogenic effects of chemicals in tissues other than the liver by the IOCA. However, the liver has been the favorite target organ studied in chemically-induced carcinogenesis (17). As more experience with different chemicals and different target tissues is gained the IOCA may become an increasingly valuable tool both for toxicologic carcinogenicity testing and for research on the mechanisms of chemical carcinogenesis.

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