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Lack of in Vivo Binding to DNA of Piroctone-olamine

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### Materials and Methods

Test compound. [6-<sup>14</sup>C]Piroctone-olamine monoethanolamine salt (I) (1-hydroxy-4-methyl-6-(2',4',4'-trimethylpentyl)-2(1H)-pyridone monoethanolamine salt) was prepared by the Hoechst company with a specific radioactivity of 33.4 mCi/mmol. The radiolabel was in the pyridone ring. The radiochemical purity was shown to be 98.5% by thin layer chromatography on polyamid 11 F<sub>254</sub> plates (Merck) with methanol/ethanolamine (99+1 vol.)

Animals. Young male SPF Wistar rats were obtained from Ivanovas, Kissleg, FRG, with weights ranging between 150 and 170 g. They were held 4 per macrolone cage on saw dust. They were fed laboratory chow (No. 21-343-7, Klingenthal Mühle AG, Kaiseraugst, Switzerland) ad libitum and they had free access to tap water for one week for acclimatisation.

Treatment. 35 mg/kg b.w. (3.5 mCi/kg) Piroctone-olamine, dissolved in 50 % aqueous 1,2-propanediol (12 mg/ml), was administered to three rats by subcutaneous injection in order to simulate 100% dermal absorption. The animals were placed single in glass metabolism cages where an air stream of 0.4 l/min transported the exhaled air to a trap with ethanolamine/methanol (1+4 vol.) to collect the expired [<sup>14</sup>C]O<sub>2</sub>.

Isolation of chromatin. 48 hours after the administration, the animals were bled by open heart puncture under ether anaesthesia. The livers were excised and homogenized in a teflon Potter-Elvehjem-type homogenizer at 4°C. Crude chromatin was prepared by precipitation with the non-ionic detergent Nonidet P 40 (BDH Chemicals Ltd., Poole BH12 4NN, England) [Sagelsdorff et al., 1983]. This pellet, containing about 2-3 mg DNA and 20-30 mg protein per g liver, was washed until the suspension contained <4000 dpm/ml.

Isolation of DNA and chromatin protein. The chromatin pellet was homogenized further in a Waring blender in 1 % (w/v) SDS, 10 mM EDTA, 8 M Urea in 0.24 M  $\text{Na}_2\text{PO}_4$ , pH 6.8. The homogenate was deproteinized with chloroform/isoamyl alcohol/phenol (CIP) and the DNA was purified by adsorption on a hydroxylapatite column, dialysis, and repetitive precipitation with ethanol to constant specific radioactivity [Sagelsdorff et al., 1983]. The highly purified DNA (less than 0.2 % protein) was dissolved in 8 mM  $\text{CaCl}_2$ , 20 mM sodium succinate, pH 6.0. The amount of DNA was determined by assuming an absorbance of 20 at 260 nm for a solution of 1 mg DNA/ml. Scintillation counting was performed after addition of 10 ml Insta-Gel in a Packard scintillation counter Tricarb 460 CD, equipped with and calibrated for the automatic analysis of labelled samples.

Chromatin protein was precipitated with acetone from the CIP extract and redissolved in 1% (w/v) SDS (five times). The last solution was diluted to 0.1% SDS and 1 ml was used for the scintillation counting. The amount of protein was determined with the Folin reagent.

Control experiments. (1) DNA was isolated from an untreated animal held together with the treated ones. The respective total count - upon comparison with historical controls - was used to show that the work-up of the DNA samples was performed without external contamination with radiolabels.

(2) The chromatin pellet isolated from the liver of an untreated rat was incubated for 15 minutes at 4°C with the radiolabelled supernatant of the first chromatin precipitation step of the DNA preparation from a treated animal. The radioactivity of the DNA isolated after this in vitro incubation was deducted from the net DNA radioactivity in the main experiment so that all radiolabel introduced into DNA only during the isolation procedure was accounted for.

## Results and Discussion

The excretion of radiolabel within 24 hours after subcutaneous injection amounted to  $6 \pm 2$  percent and  $29 \pm 10$  percent, in urine and faeces, respectively. Between 24 and 48 hours after the administration additional 4 percent and 24 percent were excreted in urine and faeces, respectively.

At this time DNA and chromatin protein was isolated from the liver and counted for [ $^{14}\text{C}$ ]-labelling. Table 1 compiles the results. The DNA samples were radiolabelled at the limit of detection. Under the preliminary assumption that this minute DNA radioactivity was due to DNA-bound Piroctone-olamine metabolites, the specific activity of the DNA samples were divided by the dose administered and converted to the molar units of the 'Covalent Binding Index, CBI', as defined in a footnote to Table 1 [Lutz, 1979]. Values of 0.016, 0.022 and 0.012 were calculated for the three treated rats.

Radioactivity on the DNA isolated from an animal that has been treated with a radiolabelled substance is not necessarily due to covalent interactions of the test compound with DNA. Non-covalent interaction of the test compound with DNA is unlikely because the data of the respective control experiments given in the third column of Table 1 show that this contribution was not measurable. A more likely reason for the observed DNA radioactivity is the biosynthetic incorporation of radioactivity if the compound is degradable to small molecules able to enter the pool of nucleic acid precursors. Such is obviously the case with compounds that are degraded to yield [ $^{14}\text{C}$ ] $\text{O}_2$ , because, for instance, the carbon 6 of the purine bases adenine and guanine is taken from a  $\text{CO}_2$  molecule. Table 2 summarizes the data on the [ $^{14}\text{C}$ ] $\text{O}_2$  exhalation after administration of [ $^{14}\text{C}$ ]Piroctone-olamine. On average, as little as 0.01 percent of the radioactivity dose administered was exhaled within 48 hours in the form of [ $^{14}\text{C}$ ] $\text{O}_2$ . This extremely small amount may be due to radiochemical impurities administered and does not necessarily mean that the pyridone moiety of Piroctone-olamine is broken down during metabolism.

Due to the finding that [ $^{14}\text{C}$ ] $\text{O}_2$  is formed after administration of the radiolabelled sample, the radioactivity in the DNA must in part be due to biosynthetic incorporation of radiolabel. Control experiments with [ $^{14}\text{C}$ ]methanol show that this source of radioactivity might fully

account for the DNA radioactivity: Exhalation of  $[^{14}\text{C}]\text{O}_2$  and biosynthetic incorporation into liver DNA were determined after oral administration of 15 mg/kg b.w.  $[^{14}\text{C}]\text{methanol}$  to male rats. DNA isolated from liver 48 hours after administration, was clearly radiolabelled and the specific activity corresponded to an apparent CBI of 428. The corresponding  $[^{14}\text{C}]\text{expiration}$  accounted to 85% of the administered radioactivity dose, exhaled as  $[^{14}\text{C}]\text{O}_2$  within 48 hours.

For an estimation of the relationship between  $[^{14}\text{C}]\text{O}_2$  exhalation and biosynthetic incorporation of radioactivity into DNA, the ratio of specific DNA activity (CBI units) per  $[^{14}\text{C}]\text{O}_2$  expired was calculated on the basis of the methanol data to be  $428:85\% = 503$  for liver DNA. Multiplication of the  $[^{14}\text{C}]\text{O}_2$  data obtained with Piroctone-olamine with this ratio resulted in an apparent Binding Index of about 0.05, i.e. in the same range as the observed DNA radioactivities expressed in CBI units.

Contamination of DNA with protein could also contribute to the observed DNA radioactivity but a quantitative analysis on the basis of the protein radioactivity given in Table 1 is complicated by the fact that precipitation of protein in the presence of high activities of small molecules must result in overestimations.

Based on the above results it is highly probable that the DNA radioactivity detected after administration of  $[^{14}\text{C}]\text{Piroctone-olamine}$  is not due to DNA binding but to biosynthetic incorporation of radiolabelled precursors of DNA synthesis and perhaps to contamination of the DNA with protein.

If one disregards the above explanation for the origin of the radioactivity on DNA and takes hypothetically the values given in Table 1 as due to covalent binding of Piroctone-olamine to DNA, the figures would be extremely low upon comparison with known carcinogens. The strongest hepatocarcinogenic compounds with a genotoxic, initiating mode of action, like aflatoxin  $\text{B}_1$  or dimethylnitrosamine, exhibit a CBI in the order of  $10^3$  to  $10^4$ . Moderate hepatocarcinogens like 2-acetylaminofluorene and vinyl chloride show a CBI of a few hundred, and weak genotoxic carcinogens such as N,N-dimethyl-4-aminoazobenzene bind to DNA on CBI level of 1-10. The maximum possible DNA-binding ability of Piroctone-olamine therefore is too low to consider DNA binding as a mechanism of putative tumorigenic action.

References

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Sagelsdorff, P., Lutz, W.K. and Schlatter, Ch., 1983. Carcinogenesis, in press.

Table 1. Radioactivity in liver DNA isolated from male rats 48 hours after subcutaneous administration of [ $^{14}\text{C}$ ]Piroctone-olamine.

Treatment	Piroctone-olamine			Control experiments	
	Rat 1	Rat 2	Rat 3	Binding in vitro	Untreated animal
Animal weight [g]	246	241	242	235	233
Chemical dose [mg/kg]	35	34	31	-	-
Radioactivity dose [dpm/kg]	$7.9 \cdot 10^9$	$7.7 \cdot 10^9$	$7.0 \cdot 10^9$	-	-
DNA:					
Amount in vial [mg]	8.5	6.6	8.2	6.0	4.6
Gross activity [cpm] $\pm 1$ S.D. <sup>a</sup>	15.6 $\pm 0.7$	15.7 $\pm 0.7$	14.7 $\pm 0.7$	13.0 $\pm 0.1$	12.9 $\pm 0.1$
Net activity [dpm] $\pm 1$ S.D. <sup>b</sup>	3.5 $\pm 1.0$	3.6 $\pm 1.0$	2.3 $\pm 1.0$	-	-
Specific activity [dpm/mg] $\pm 1$ S.D.	0.4 $\pm 0.1$	0.6 $\pm 0.2$	$\pm 0.3$ $\pm 0.1$	-	-
[CBI units] $\pm 1$ S.D. <sup>c</sup>	0.016 $\pm 0.005$	0.022 $\pm 0.006$	0.012 $\pm 0.005$	-	-
Control Data					
Crude chromatin protein [dpm/mg]	157	133	113	-	-

- <sup>a</sup> The total variability (statistical counting error and fluctuations due to vial, scintillation cocktail, counter, external radiation) for a DNA sample containing little radioactivity was assumed to be equal to the variability of DNA samples isolated from untreated animals held together with the treated ones. On the basis of 36 background values compiled for 2 years, a respective standard deviation of 0.73 cpm was calculated. The standard deviation for a net radioactivity in a vial therefore was taken as:

$$1 \text{ S.D.} = \sqrt{0.73^2 + (0.73/\sqrt{36})^2} = 0.74 \text{ cpm}$$

- <sup>b</sup> The limit of detection for radioactivity in a vial was calculated on a level of 2 S.D. The counting efficiency was 75%

- <sup>c</sup> The Covalent Binding Index (CBI) is defined as:

$$\text{CBI} = \frac{\mu\text{mol chemical bound} / \text{mol DNA nucleotide}}{\mu\text{mol chemical applied} / \text{kg body weight}}$$

$$\text{CBI} = \frac{\text{dpm} / \text{mg DNA}}{\text{dpm} / \text{kg body weight}} \cdot 3.00 \cdot 10^8$$

Table 2. Exhalation of [ $^{14}\text{C}$ ]labelled  $\text{CO}_2$  after subcutaneous administration of [ $^{14}\text{C}$ ]Piroctone-olamine to 3 male rats

Period of collection [h after application]	Cumulative exhalation		
	Fraction of dose [ $10^{-5}$ ]		
	Rat 1	Rat 2	Rat 3
0 - 4	2.4	2.8	3.0
4 - 10	3.0	3.6	4.6
10 - 20	4.6	6.1	8.4
20 - 30	5.6	8.0	11.0
30 - 48	6.5	9.7	13.0