Europ. J. Cancer Vol. 16, pp. 1283–1287
© Pergamon Press Ltd. 1980. Printed in Great Britain

0014-2964/80/1001-1283 $02.00/0

Reactivity of the *O,O'*-Diacetyl Derivative of the Carcinogen 4-Hydroxyaminoquinoline-1-Oxide with DNA. Comparison with *in Vivo*-Reacted DNA*

S. GALIÈGUE, B. BAILLEUL and M.-H. LOUCHEUX-LEFEBVRE†

Institut de Recherches sur le Cancer de Lille et Unité 124 *de l'INSERM, BP* 311, 59020 *Lille Cédex, France*

Abstract—*An ester of* 4-*hydroxyaminoquinoline*-1-*oxide* (4-*HAQO*), *the O,O'-diacetyl derivative* (*diAc*-4-*HAQO*), *was previously considered as a possible ultimate metabolite of the carcinogen* 4-*nitroquinoline*-1-*oxide* (4-*NQO*). *In order to verify such a hypothesis we have carried out a fluorescent comparative study between the* in vitro *diAc*-4-*HAQO reacted DNA and the* in vivo 4-*HAQO and* 4-*NQO modified DNA. Our results show that diAc*-4-*HAQO could be used as a model of ultimate carcinogen of* 4-*NQO.*

INTRODUCTION

THE POTENT carcinogenic action of 4-nitroquinoline-1-oxide (4-NQO) is well known since it was first demonstrated by Nakahara *et al.* [1]. Later, it was proposed that 4-hydroxyaminoquinoline-1-oxide (4-HAQO), which is a metabolite of 4-NQO, might be the proximate carcinogen [2] and that the reductive pathway which yields 4-HAQO seemed to be essential for the carcinogenic mechanism of 4-NQO [3, 4]. More recently, Tada and Tada [5] have shown that an unique enzymatic system could be responsible for the activation of 4-HAQO.

By analogy with other carcinogens, such as acetyl-aminofluorene [6], it was considered that esters of 4-HAQO could appear as ultimate metabolites in the carcinogenic activity. Indeed, Enomoto *et al.* [7] reported that the *O,O'*-diacetyl derivative of 4-HAQO (diAc-4-HAQO) reacted covalently and non-enzymatically with DNA at neutrality. The diAc-4-HAQO-reacted DNA exhibits particular fluorescent properties and preliminary work was carried out by Enomoto *et al.* [7] in order to compare these fluorescent properties with those reported by Tada *et al.* [8] and Matsushima *et al.* [9] for the reacted nucleic acid formed *in vivo* by exposure of ascite cells to 4-HAQO. The work of Enomoto *et al.* [7] is the only publication to date which concerns the *in vitro* binding of the diacetyl derivative with DNA and unfortunately, no spectra are shown in this work. It is therefore impossible to appreciate the degree of accuracy of such a comparison.

In this paper we carry out a comparative fluorescence study between the *in vivo*- and *in vitro*-reacted DNA, with either 4-NQO and 4-HAQO or diAc-4-HAQO respectively. In the *in vivo* experiments the complexes between carcinogen and DNA were formed by exposure of Zajdela ascite tumor cells to 4-HAQO and 4-NQO. The *in vitro* diAc-4-HAQO reacted DNA was obtained by using the ascite cell DNA.

In the two cases, the determination of quinoline binding DNA was carried out by using 4-HA[2-^{3}H]QO and diAc-4-HA[2-^{3}H]QO.

MATERIALS AND METHODS

Chemicals

4-NQO was kindly provided by Professors Sato and Kawazoe.

4-HAQO was obtained from 4-NQO by the method of Enomoto *et al.* [7].

DiAc-4-HAQO was prepared from 4-HAQO according to the method of Kawazoe and Araki [10].

Accepted 3 March 1980.
*This work was supported by the Délégation Générale á la Recherche Scientifique et Technique" (Grant No. 78.7.2628).
†To whom correspondence should be addressed.

4-N[2-^{3}H]QO was obtained from Amersham Ltd. (specific radioactivity 941 mCi/mmole).

4-HA[2-^{3}H]QO obtained as previously described [7] presented a specific radioactivity of 18.42 mCi/mmole.

DiAc-4-HA[2-^{3}H]QO obtained from the method of Kawazoe and Araki [10] exhibits a specific radioactivity of 9630 counts/min/nmole.

Preparation of in vivo-*reacted DNA*

The method of preparation was previously described by Ikegami *et al.* [11]. Approximately 10^6 ascite tumor cells (H.Z. line) were injected i.p. into 3-month-old male Wistar rats of about 300 g. Seven days after transplantation, either 4-NQO or 4-HAQO was i.p. injected.

For 4-NQO, a 0.018 M solution was used. It was obtained from 4-NQO dissolved in a small amount of dimethylsulfoxide followed by appropriate dilution with physiological saline solution. For 4-HAQO the 0.03 M solution was obtained by direct dissolution of the carcinogen in physiological saline solution. In the two cases 0.5 ml/rat of the carcinogen solution was injected. The rats were killed by decapitation either 1 or 2 hr later. The ascitic fluid was drained and the cells were harvested by centrifugation (5 min at 1500 rev/min). Cells were washed in a 0.25 M/0.88 M sucrose gradient containing 10% PBS in order to remove contaminating red blood cells. Then DNA was extracted using the Marmur's method [12].

Preparation of in vitro-*reacted DNA*

The DNA was obtained from ascite cells of untreated rats using the method of extraction of Marmur [12]. DNA in a 2×10^{-3} M sodium citrate buffer pH 7 was reacted with diAc-4-HAQO dissolved in EtOH in order to obtain a final buffer/EtOH ratio: 80/20 by volume. The initial reaction mixture is characterized by R, the ratio of molar concentration of diAc-4-HAQO/concentration of DNA expressed in mole P/l. Many samples of modified DNA's were prepared corresponding to different values of R. The reaction is carried out at 37°C, in the darkness, for 45 min. After reaction, the modified DNA was purified by extensive diethyl ether extractions. The remaining non-covalently bound quinoline residues were removed by extensive precipitations and washing with ethanol. The lack of non-covalently bound quinoline was checked by counting aliquots of 200 µl of the supernatant ethanolic fractions.

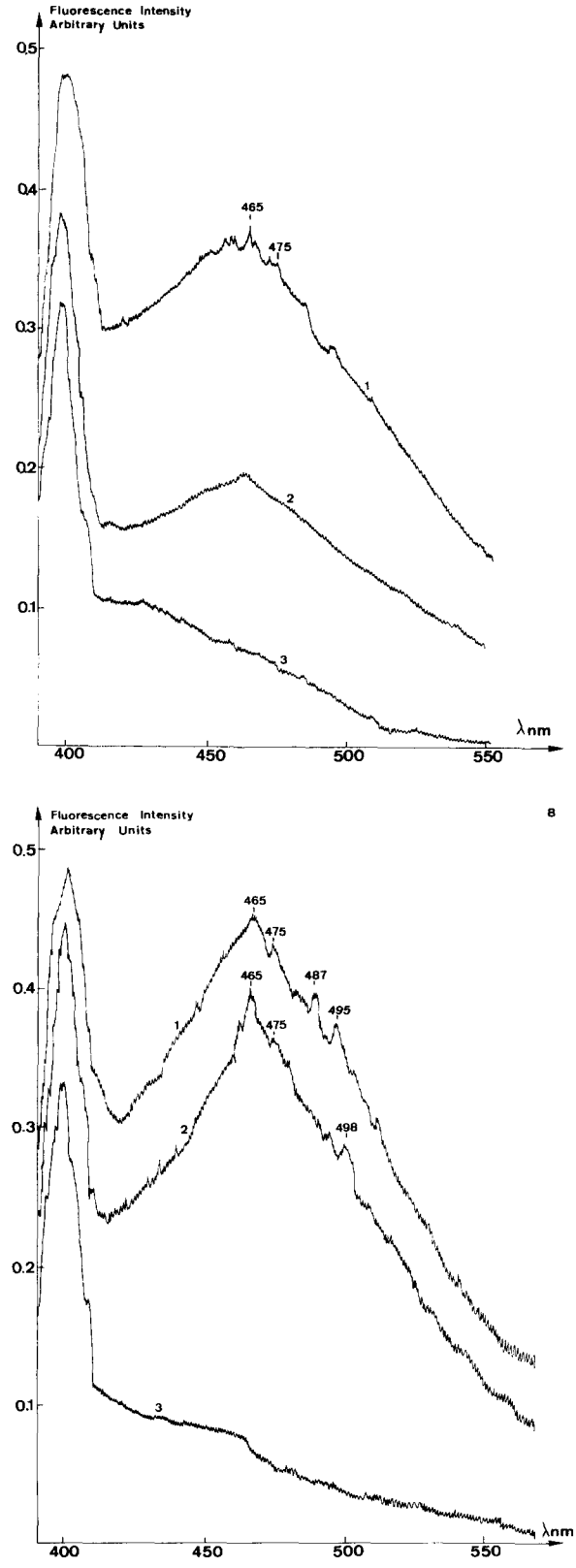

Fig. 1. Fluorescence spectra of DNA isolated from (A) 4-NQO and (B) 4-HAQO-exposed ascite tumor cells, in 2 $\times 10^{-3}$M citrate buffer, pH 7. *The concentration of DNA was* 1 mg/ml *and the excitation wavelength* 350 mm. *The figure shows the corrected readings at different exposure times. Curve* 1: t = 2 hr; *curve* 2: t = 1 hr; *curve* 3: *no carcinogen exposure (reference DNA). The band at* 400 nm *is corresponding to a Raman vibration of water.*

Fluorescence studies

The fluorescence spectra were recorded with a differential spectrofluorimeter (model FICA 55 000). The apparatus is totally corrected, that is, in excitation (the fluctuations and the ageing of the lamp are automatically compensated) and in emission. The spectra were recorded using the unreacted DNA as reference. Fluorescence spectra were measured in 2×10^{-3} M citrate buffer pH 7. Concentration of DNA was 1 mg/ml for the *in vivo* reacted DNA.

The fluorescence curves were recorded in arbitrary units; therefore the quantitative determinations were impossible; more especially the quantum yield of the bound carcinogen could not be calculated. Nevertheless, a comparison between the intensities of different fluorescence spectra could be carried out when these spectra were recorded with exactly the same experimental conditions and with identical settings on the spectrofluorimeter.

RESULTS

Fluorescence studies

In vivo-*reacted DNA*. DNA isolated from 4-NQO- and 4-HAQO-exposed cells is fluorescent. The spectra obtained with an excitation wavelength at 350 nm (Fig. 1) exhibit a fluorescence emission band with a main maximum at 465 nm either for 4-NQO- or 4-HAQO-modified DNA. In the case of 4-HAQO-modified DNA, the spectrum is slightly structured and three secondary maxima at 475, 487 and 495 nm are observed. It appears that the fluorescence intensity and consequently the amount of bound fluorescent compound depends on the time of carcinogen exposure, essentially in the case of 4-NQO. As observed in Fig. 1, the spectrum is more intense after 2 hr than after 1 hr of exposure. For 4-HAQO-modified DNA, the difference

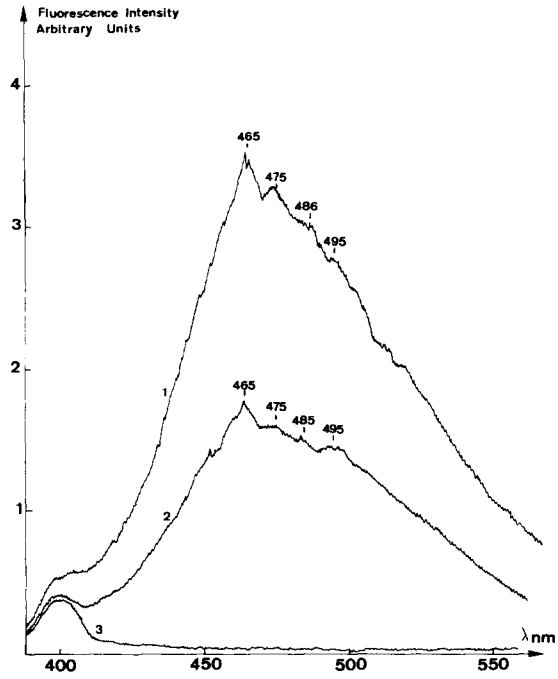

Fig.2. Fluorescence spectra of the diAc-4-HAQO-reacted DNA. The DNA was isolated from untreated ascite tumor cells. The spectra were recorded with 0.3 mg/ml *DNA solutions in* 2 $\times 10^{-3}$ M *citrate buffer*, pH 7. *The excitation wavelength was* 350 nm. *The figure shows the corrected instrument readings for different values of R, ratio of molar concentration of diAc-4-HAQO/concentration of DNA expressed in* mole P/l, *in the initial reaction mixture. Curve* 1: $R=0.5$; *curve* 2: $R=0.2$; *curve* 3: $R=0$ (*reference DNA*). *The band at* 400 nm *has the same meaning as that in Fig.* 1.

observed between the two spectra obtained after 1 or 2 hr of exposure of cells is not significant.

In vitro-*reacted DNA*. The fluorescence spectra obtained with an excitation at 350 nm are shown in Fig. 2. Two spectra corresponding to two different values of R (R = 0.5; R = 0.2) are shown. These spectra are slightly structured and exhibit a principal maximum at 465 nm and three secondary maxima at 475, 485 and 495 nm.

Table 1. *Determination of the quinoline binding to DNA in* vivo *experiments using* 4-*HA*[2-^{3}H]*QO*. Half a ml *of the solution of carcinogen in physiological saline solution was injected i.p. to rats bearing a Zajdela ascite tumor. The exposure time was* 1 *and* 2 hr *and two rats were used for each experiment*

	Time after carcinogen injection (hr)	DNA specific activity counts/min/mg DNA	Quinoline binding to DNA, expressed as molecule of carcinogen per nucleotide
4-HA[2-^{3}H]QO	1	3333	$5{,}4 \times 10^{-5}$
0.03 M	1	2920	$4{,}7 \times 10^{-5}$
4-HA[2-^{3}H]QO	2	2714	$4{,}6 \times 10^{-5}$
0.006 M	1	600	1×10^{-5}

Determination of quinoline binding to DNA

In vivo-*reacted DNA*. The extent of binding of isotope to DNA at 1 and 2 hr after injection of 4-HA[2-^{3}H]QO is given in Table 1. Moreover, we show that by using different amounts of 4 HAQO (30 mM and 6 mM), the percentage of quinoline binding to DNA is proportional to the injected quantity of carcinogen. As observed in Table 1, the specific activity of DNA obtained with the more concentrated solution of 4-HAQO—either for 1 or 2 hr of exposure—corresponds to approximately 1 molecule of quinoline derivative per 20,000 nucleotides of DNA. This result is in good agreement with those previously obtained by Ikegami *et al.* [11] and Tada and Tada [13].

In vitro-*reacted DNA*. Many samples corresponding to different extents of carcinogen binding to DNA were characterized (from 0.2 to 1.0 quinoline ring per 100 nucleotides). The details concerning these determinations will be given elsewhere.

DISCUSSION

Since the fluorescent properties of reacted DNA were essentially due to the quinoline ring one can claim that the quinoline moiety is covalently bound to DNA either *in vitro* with diAc-4-HAQO or *in vivo* from 4-NQO and 4-HAQO. As expected, the percentage of bound rings is higher in the *in vitro* than in the *in vivo* reactions.

For both *in vivo* and *in vitro* reacted DNA the fluorescence spectra exhibit a principal maximum at the same wavelength i.e., 465 nm and, in the two cases, three additional maxima at 475, about 485 and 495 nm are observed. Such characteristically similar fluorescence spectra support the proposal that diAc-4-HAQO constitutes a valid model for the ultimate carcinogen in investigating chemical carcinogenesis by 4-NQO.

This result, however, does not allow us to state that the reaction was exactly the same in the two cases, i.e., that the adducts are identical. Further work is in progress in our laboratory to characterize these adducts.

Acknowledgements—The authors would like to express appreciation to Pr. Gérard Biserte, Director of the Institut de Recherches sur le Cancer de Lille, for discussions and advice. Thanks are also due to Mrs M. N. Vlaeminck and Miss A. M. Debévère for technical assistance concerning the *in vivo* experiments. The fluorescence studies were carried out at the Université des Sciences et Techniques de Lille with the help of Dr. A. Pollet.

One of us (S.G.) is a recipient of a fellowship from the Ligue Nationale Francaise contre le Cancer.

The authors are grateful to Dr. B. M. Carden for critical reading of the English text.

REFERENCES

1. W. Nakahara, F. Fukuoka and T. Sugimura, Carcinogenic action of 4-nitroquinoline-*N*-oxide. *Gann* **48,** 129 (1957).
2. Y. Shirasu, Further studies on carcinogenic action of 4-hydroxyaminoquinoline-1-oxide. *Gann* **54,** 487 (1963).
3. T. Sugimura, K. Okabe and H. Endo, The metabolism of 4-nitroquinoline-1-oxide. I. Conversion of 4-nitroquinoline-1-oxide to 4-aminoquinoline-1-oxide by rat liver enzymes. *Gann* **56,** 489 (1965).
4. T. Sugimura, K. Okabe and M. Nagao, The metabolism of 4-nitroquinoline-1-oxide, a carcinogen. III. An enzyme catalyzing the conversion of 4-nitroquinoline-1-oxide to 4-hydroxyaminoquinoline-1-oxide in rat liver and hepatomas. *Cancer Res.* **26,** 1717 (1966).
5. Mit. Tada and Mar. Tada, Seryl-tRNA synthetase and activation of the carcinogen 4-nitroquinoline-1-oxide. *Nature* (*Lond.*) **255,** 510 (1975).
6. J. A. Miller, Carcinogenesis by chemicals: an overview—G.H.A. Clowes memorial lecture. *Cancer Res.* **30,** 559 (1970).
7. M. Enomoto, K. Sato, E. C. Miller and J. A. Miller, Reactivity of the diacetyl derivative of the carcinogen 4-hydroxyaminoquinoline-1-oxide with DNA, RNA and other nucleophiles. *Life Sci.* **7,** (part II), 1025 (1968).
8. Mar. Tada, Mit Tada and T. Takahashi, Interaction of a carcinogen, 4-hydroxyaminoquinoline-1-oxide with nucleic acids. *Biochem. biophys. Res. Comm.* **29,** 469 (1967).
9. T. Matsushima, I. Kobuna and T. Sugimura, *In vivo* interaction of 4-nitroquinoline-1-oxide and its derivatives with DNA. *Nature* (*Lond.*) **216,** 508 (1967).
10. Y. Kawazoe and M. Araki, Studies on chemical carcinogenesis. V. *O,O'*-diacetyl-4-hydroxyaminoquinoline-1-oxide. *Gann* **58,** 485 (1967).

11. S. IKEGAMI, N. NEMOTO, S. SATO and T. SUGIMURA, Binding of ^{14}C-labeled 4-nitroquinoline-1-oxide to DNA *in vivo*. *Chem. biol. Interact.* **1,** 321 (1969/1970).
12. J. MARMUR, A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. mol. Biol.* **3,** 208 (1961).
13. Mit. TADA and Mar. TADA, Interaction of a carcinogen, 4-nitroquinoline-1-oxide, with nucleic acids: chemical degradation of the adducts. *Chem. biol. Interact.* **3,** 225 (1971).