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Tamoxifen-induced DNA adducts in leucocytes of breast cancer patients

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Tamoxifen-induced DNA adducts were searched in leucocyte DNA from breast cancer patients. Total white blood cell DNA from tamoxifen-treated and control patients was analysed by ^{32}P-postlabelling using HPLC-radioactivity detection. Rat liver DNA was used as a positive standard. In blinded analysis four of the six treated patients showed DNA adducts; none of the five controls were positive. The identity of fraction as a tamoxifen adduct was confirmed by using different chromatographic systems, each with spiked rat liver samples. The level of adducts in the treated patients was 5.5 adducts/10^9 nucleotides as compared to an apparent level of 1.9/10^9 in the controls.

Introduction

Tamoxifen is a nonsteroidal antiestrogen successfully used in adjuvant therapy of breast cancer since the 1970s. It has been estimated that recurrence of breast cancer is decreased by 40% due to long-term adjuvant treatment (1). Several studies are currently being carried out in assumed risk populations where tamoxifen is used prophylactically. Tamoxifen has been shown to cause liver cancer (2–5) and DNA adducts in several organs in experimental animals (6–10) and cultured human lymphocytes and human microsomes *in vitro* (11–13). Endometrial cancer and other serious side-effects of therapy have been reported in tamoxifen-treated patients (1, 14–16). In 1996 a working group of the International Agency for Research on Cancer (IARC) concluded that tamoxifen is carcinogenic to humans (17). It is thus of large mechanistic interest to investigate the levels of DNA adducts in humans and their possible utility in risk assessment. However, the studies so far reported have found no adducts in humans (18–20).

Considering the expected low level of adducts and interference from background we apply here the ^{32}P-postlabelling method coupled to high-performance liquid chromatography (HPLC*) and radioactivity detection (21) to leucocyte samples from breast cancer patients receiving tamoxifen and controls. Adducts comigrating with those from tamoxifen-treated rat livers are demonstrated.

Materials and methods

Patients, DNA isolation, rat liver standard

Blood samples (10 ml) were obtained from 11 breast cancer patients. Of these six (mean age 72 years) continuously received tamoxifen, 40 mg/day orally, for a minimum of 4 months, and five (mean age 51 years) had not been given the drug (Table I). All women, with the exception of one 90 year old in the treated group, had been operated. The samples were coded for blinded analysis and stored at –20°C. DNA was isolated by first lysing the cells with 0.5% Triton X and recovering the crude nuclear fraction. The nuclear fraction was then treated with RNase A and T1, followed by proteinase K and extraction with phenol and chloroform–isoamyl alcohol. DNA was precipitated and washed with ethanol. This procedure ensured efficient removal of RNA, as evident in the HPLC analysis of the labelled products (see below).

Rat liver DNA was used as a standard in the analyses. The animals had received tamoxifen p.o. 45 mg/kg. The DNA isolation was carried out as previously described (11). In brief, the rat liver (0.6–1.2 g) was homogenised in 5 ml of 1 mM $MgCl_2$, 10 mM Tris–HCl, 0.15 M NaCl (pH 8), after which the suspension was treated with 5 ml of 0.5% Triton X-100. The nuclei were collected by centrifugation at 3000 rpm for 10 min at 20°C. DNA was isolated with RNase A and RNase T1 treatment followed by proteinase K digestion, as described above.

Labelling and separation

DNA (10 μg) was enzymatically digested to 3′-mononucleotides as described (11), first by incubating DNA for 2 h at 37°C with micrococcal nuclease (80 mU/mg DNA in 3 mM bicine pH 9.0, 0.5 mM $CaCl_2$) and then for 2 h at 37°C with spleen phosphodiesterase (1.6 mU/mg DNA) in added 20 mM ammonium acetate, pH 5.0. P1 nuclease (1 mg/ml; 1 h at 37°C) was used for dephosphorylation of normal nucleotides.

The modified nucleotides were converted to ^{32}P-postlabelled diphosphates in labelling mixture (2 μl) containing 2.4 U T4 polynucleotide kinase and 2.3 pmol ATP (7 μCi [g-^{32}P]ATP, 3000 Ci/mmol). The reaction was carried out at pH 9.6.

For HPLC analysis the mixtures were diluted to 20 μl with water, and the whole sample was injected into the Beckman HPLC system Gold, used with a Phenomenex Kromasil C18 (2×250 mm, particle size 5 μm) column. A precolumn filter was installed in front of the analytical column. The volume of the sample loop was 20 μl.

Radioactivity was measured on-line with a Beckman 171 Radioisotope detector. The size of the teflon sample loop in the flow cell was 75/100 μl, which was then folded into a scintillation tube containing scintillation liquid (Ready Safe, Beckman). The adducts were quantified by integration of the peak area with subtraction of background radioactivity.

Separations were carried out at ambient temperature using a binary gradient with methanol and 0.2 M ammonium formate (pH 5.4) adjusted to pH 4.2 with phosphoric acid (resulting in a final concentration of 20 mM with respect to phosphoric acid). Labelled samples were analysed using two different gradients. Gradient A had initial conditions of 2% methanol for 5 min after which the proportion of methanol increased linearly to 70% in 65 min and then further to 100 % in 5 min. 100% methanol was maintained for 10 min before linear decrease to 2% in 10 min. In Gradient B the proportion of methanol increased to 40% in 40 min and then to 45% in 10 min and it was maintained for 15 min at which time the tamoxifen adduct eluted. After that methanol concentration was increased to 100% in 20 min. Flow rate was 0.25 ml/min.

Thin-layer chromatography (TLC) analyses were performed on 10×10 cm polyethyleneimine (PEI)-cellulose TLC plates (Macherey-Nagel, Duren, Germany) as described (11). When HPLC fractions were analysed the plates were developed only in directions 2 and 3. The plates were washed by flotation in water between each dimension. The adducts were detected in a Fuji XBAS 2000 phosphorimager.

Analysis of results

Adducts levels were calculated from the radioactivity of the samples, background subtracted, assuming 100% recovery and labelling efficiency. As these never are 100% (22,23), the ‘apparent’ adduct levels are underestimates. In the HPLC analysis the background was obtained from an untreated human sample, analysed exactly as the other samples. The approximate detection limit using such a background definition was 1 adduct/10^9 nucleotides in the HPLC analysis. For evaluation of statistical significance, Student’s one-sided *t*-test was used, by considering equality of variance. *F*-statistics was also applied.

***Abbreviations:** HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

Table I. Information relating to patients

Patient	Age	Smoking status	Operation date	Tamoxifen treatment[a] (mo)
1	45	nonsmoker	931104	17[b]
2	78	exsmoker	941011	7
3	60	smoker	940502	15
4	90	nonsmoker	not operated	4
5	77	nonsmoker	931122	21
6	83	nonsmoker	940705	14
7	36	exsmoker	950308	–
8	77	smoker	861021	–
9	51	exsmoker	950206	–
10	61	smoker	931115	–
11	31	nonsmoker	920622	–

[a]Dose 40 mg/day.
[b]Concurrent drugs: zoladex + goserelin acetate.

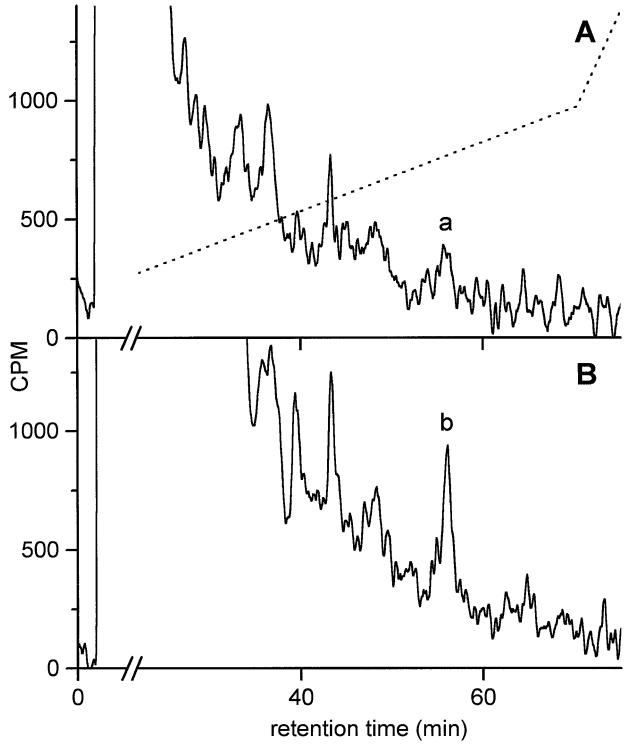

Fig. 1. HPLC-radioactivity detector analysis of human leucocyte DNA of a control (**A**) and breast cancer patient treated with tamoxifen (**B**). The area of the tamoxifen adduct is indicated as 'a' and 'b'. Gradient A in HPLC, increasing methanol concentration is indicated by a broken line in (A).

Results

HPLC analysis was performed using Gradient A and a tamoxifen adduct peak was observed at 56 min (Figure 1B). In control samples a small amount of radioactivity was observed at that elution time (Figure1A). These results were reproduced in another gradient system, Gradient B.

When a mixing experiment was carried out by spiking a liver DNA sample from a tamoxifen-treated rat (Figure 2A) with an equal radioactivity from a human leucocyte sample of a tamoxifen-treated patient (Figure 2B), a uniform peak was obtained (Figure 2C) suggesting that the material is identical. The spiked human and rat liver samples also showed identical mobility in Gradient B, in which tamoxifen adduct eluted in an isocratic part of the program. The adduct from rats and humans eluted in two adjacent main peaks, which remained uniform in the mixing experiment, providing further evidence on the identity of the material (Figure 3).

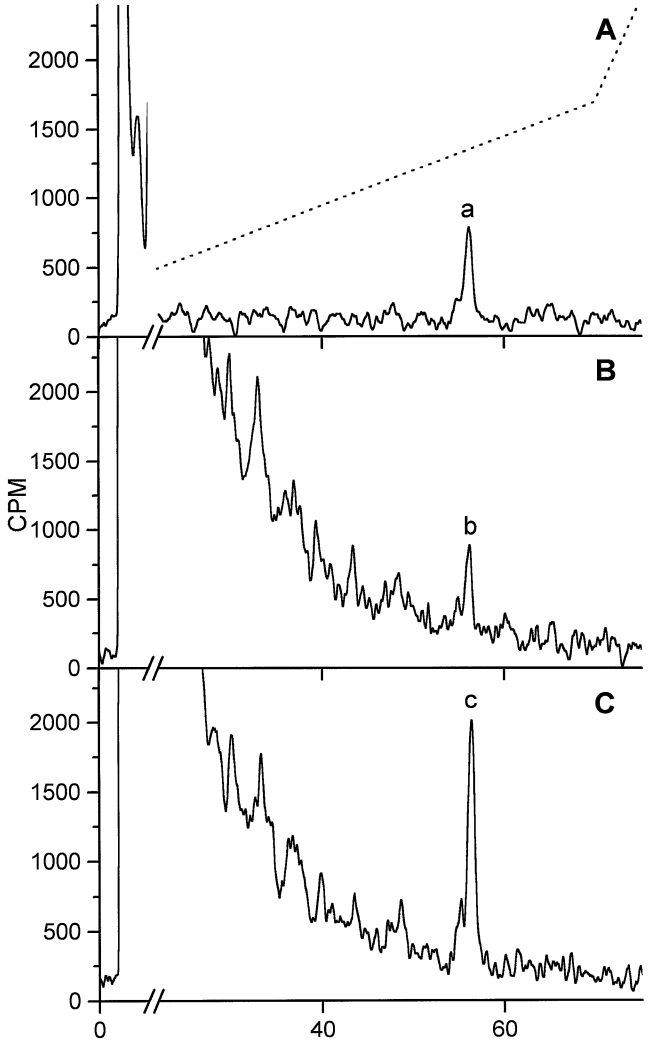

Fig. 2. HPLC-radioactivity detector analysis of rat liver DNA from a tamoxifen-treated animal (**A**), human leucocyte DNA from a tamoxifen-treated patient (**B**) and mixing of the two in equal proportions (**C**). Gradient A, increasing methanol marked by a broken line in (A).

The tamoxifen adduct peak collected from HPLC (Figure 1) was further analysed in TLC. Individual spots were obtained, and the one from a control patient was faint (Figure 4A and B). The adduct spot from a control patient 'a1' migrated identically to a faint spot 'b1' from a treated patient. The main tamoxifen adduct was 'b2' in Figure 4B. Taken together, the combined HPLC-TLC analysis indicated that true tamoxifen-induced adducts could be identified.

Among the 11 patients in the study six had received tamoxifen. When analysing coded samples by HPLC, four samples were considered clearly positive and they were all obtained from the patients who had indeed received the drug. None of the controls were considered positive. The differences between the tamoxifen-treated patients (5.5 adducts/10^9) and the controls (1.9 adducts/10^9), analysed by HPLC, are significant statistically ($P<0.05$, one-sided *t*-test or *F*-test). The value for the controls is quite arbitrary because no consistent radioactive peaks were noted in HPLC. The medians and the distribution of individual values is shown in Figure 5.

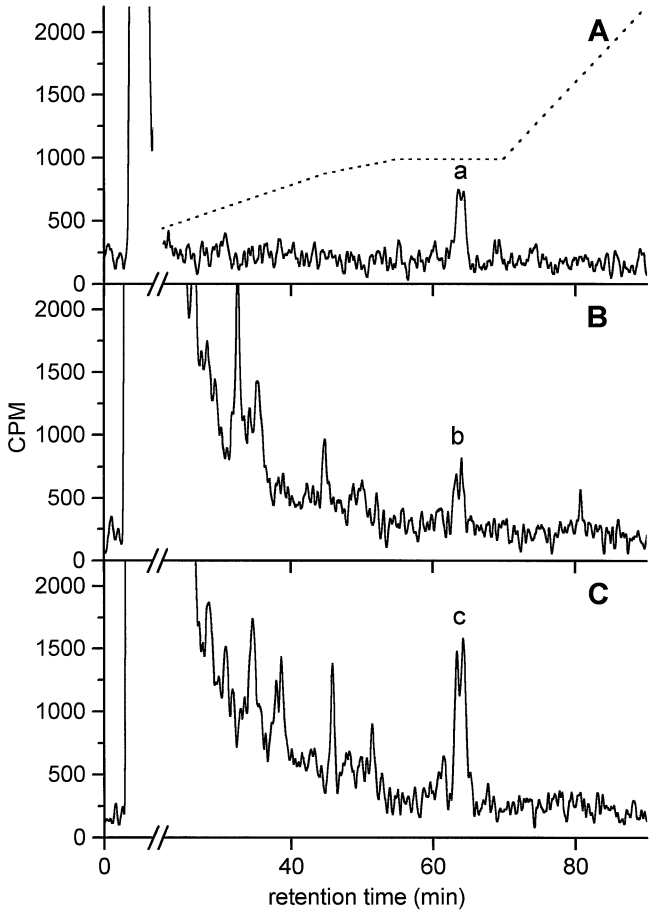

Fig. 3. HPLC-radioactivity detector analysis of rat liver DNA from a tamoxifen-treated animal (**A**), human leucocyte DNA from a tamoxifen-treated patient (**B**) and mixing of the two in equal proportions (**C**). Gradient B, increasing methanol indicated by a broken line in (A).

Discussion

This is the first report on the identification of tamoxifen-DNA adducts in human leucocytes, matching our recent data on endometrial samples (24). The identification was based initially on blinded HPLC analysis of coded samples. Of 11 patient samples four were considered positive and seven negative using the rat liver adduct as a standard. All the four were indeed treated patients. Thus no false positives but two false negatives were detected. The reason for the two patients being negative for DNA adducts remains unknown.

After opening the code several studies were carried out to confirm the result, including analysis in two HPLC systems, spiking experiments with rat liver DNA samples in two systems and HPLC-TLC analysis in two systems. These data should leave little doubt about identification of true tamoxifen adducts.

The apparent level of leucocyte adducts was $5.5/10^9$ nucleotides. In human endometrial samples, analysed by the same technique, the level of adducts was $3/10^9$ nucleotides (24). However as the adducts have not been characterised chemically and thus the total recovery of the method cannot be assessed, the present adduct level is most likely an underestimate (22,23). It is indeed an urgent task to characterise the adducts chemically, which would then allow comparisons to other types of adducts. The work on active intermediates and binding sites in DNA facilitates the final characterization (8–14, 25,26). Comparisons between species and extrapolation to carcinogenic potency will also provide important mechanistic data (27,28).

In previous work no adducts have been detected in human liver (18), leucocytes (20) nor endometrium (19). The main difference to the present work is that we used HPLC while the others used TLC in the separation of the adducts. Our HPLC method helped to reduce background radioactivity, thus boosting the effective level of detection to ~1 adduct/10^9. Although a similar sensitivity can be achieved by TLC (19–21), the background may reduce the effective sensitivity. Martin *et al*. (18) reported a background radioactivity in their

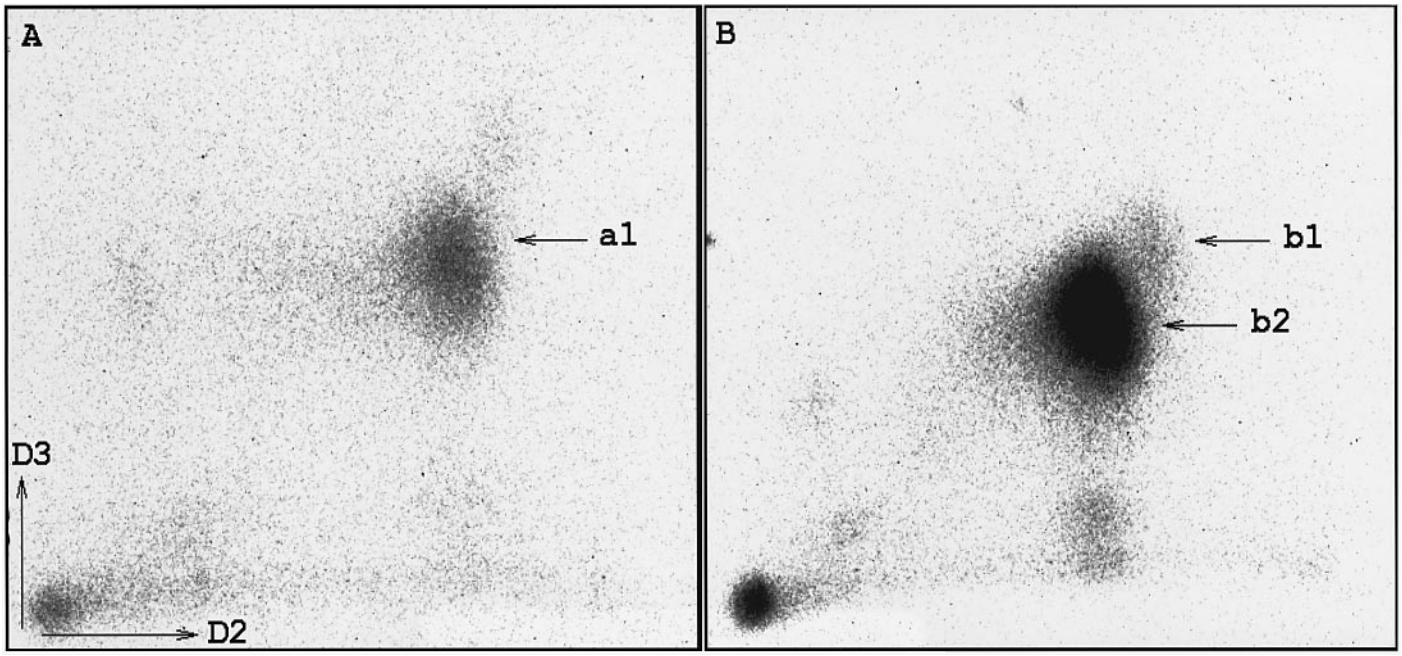

Fig. 4. TLC analysis of leucocyte DNA first analysed by HPLC (as in Fig. 1); the tamoxifen-DNA adduct fraction was collected and then analysed by TLC. (**A**) Control and (**B**) tamoxifen-treated patient; 'a1' and 'b1' migrate in the same position, 'b2' is the main tamoxifen-related adduct. The TLC plates are analysed by a phosphorimager.

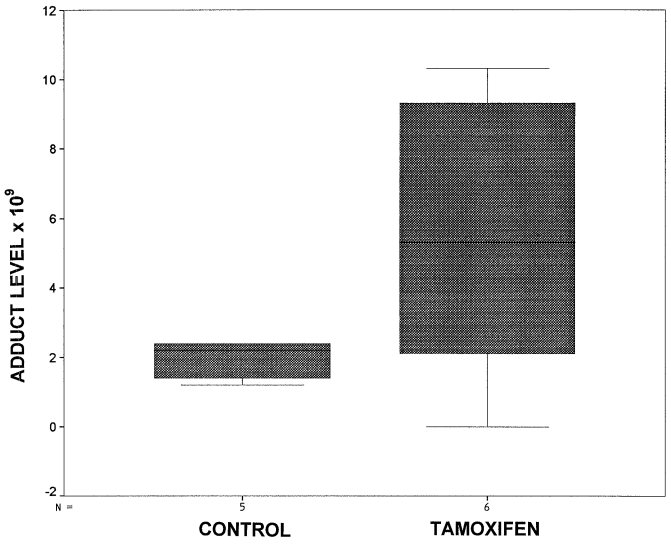

Fig. 5. Median adduct levels in tamoxifen-treated patients and controls. The horizontal line in the boxplot gives the median, the box 50% of the measurements and the horizontal lines the extreme values. The results were obtained by HPLC.

TLC analysis of 180–800 adducts/10^9, which results at a low effective detection limit. Thus the HPLC method, developed by Möller and co-workers (29,30), appears superior to TLC in analysis of clinical tamoxifen samples, although its absolute sensitivity is lower that that of TLC (21,31). Another advantage of the method is its reproducibility and applicability to a large number of clinical samples.

Tamoxifen binds to DNA causing adducts and conforming to a common mechanism by which chemicals are thought to cause mutations and cancer. However, due to its hormonal effects, tamoxifen may well have other carcinogenic mechanisms (16). Comparison of DNA forming ability in target tissues and therapeutic response between tamoxifen and other nonsteroidal antiestrogens may provide further insight in their mechanism of action.

Studies of DNA adducts in human population at the risk of cancer have suggested that the level of DNA adducts are indicative of such risks (22). Thus adduct studies in humans may also be informative regarding the cancer risks posed by therapeutic agents. Conversely, the role of DNA adducts in cancer, so far supported largely by mechanistic and circumstantial evidence, can be tested in systems such as tamoxifen treatment, if and when adducts can reliably be measured in endometrial samples.

Other applications of DNA adduct determination should be in comparative risk estimation in the search for safe drugs. As the extent of DNA adduct formation in target tissues among structural analogs may be related to their carcinogenic potency, it would be relevant to compare DNA binding properties of analogues with similar therapeutic effects.

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