Short-term dosing of $\alpha$-hydroxytamoxifen results in DNA damage but does not lead to liver tumours in female Wistar/Han rats

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It is now generally accepted that activation of tamoxifen occurs as a result of metabolism to $\alpha$-hydroxytamoxifen. In this study, $\alpha$-hydroxytamoxifen was given to female Wistar/Han rats (0.103 or 0.0103 mmol/kg, intraperitoneally, daily for 5 days). This resulted in liver DNA damage, determined by $^{32}$P-post-labelling, of 3333 ± 795 or 343 ± 68 adducts/10$^8$ nucleotides, respectively (mean ± SD, $n = 4$). Following HPLC separation, the retention times of the major $\alpha$-hydroxytamoxifen DNA adducts were similar to those seen following the administration of tamoxifen. However, after rats were treated with $\alpha$-hydroxytamoxifen (0.103 mmol/kg) for 5 days and the animals kept for up to 13 months, no liver tumours developed (0/7 rats), even with phenobarbital promotion (0/5 rats). GST-P foci were detected in the liver, but only after 13 months was their number or area significantly increased over the corresponding controls. When $\alpha$-hydroxytamoxifen was given to female $\lambda\lambda\lambda\lambda\lambda$ transgenic rats (0.103 mmol/kg orally for 10 days) and the animals killed 46 days later, there was an approximate 1.8-fold increase in mutation frequency but no significant increase in G:C to T:A transversions as described after tamoxifen treatment. It is concluded that DNA damage alone, resulting from the short-term administration of $\alpha$-hydroxytamoxifen, is not sufficient to initiate liver tumours even with phenobarbital promotion. As with tamoxifen, long-term exposure may be required to allow promotion and progression of transformed cells.

Introduction

The anti-oestrogenic drug tamoxifen is widely used as adjuvant therapy in the treatment of breast cancer in women (1). This drug may also be effective in the chemoprevention of breast cancer (2). The optimum duration of treatment has not been clearly established but results suggest at least 5 years is beneficial (3). Following long-term treatment in rats, tamoxifen is a liver carcinogen. In female Wistar rats, 11 months exposure to dietary tamoxifen (420 p.p.m.) results in about a 60% incidence of hepatocellular carcinomas (4), while in Sprague–Dawley rats, 12 months daily dosing of 22.5 mg/kg tamoxifen results in 100% incidence of liver tumours (5). The time to tumour is shortened by the promoting effects of phenobarbital in the drinking water (6). Tamoxifen has to undergo activation by CYP-dependent monoxygenases to give an electrophile that binds to DNA (7). A number of active intermediates have been proposed, but it is now generally accepted that activation occurs as a result of metabolism of tamoxifen first to $\alpha$-hydroxytamoxifen that is then converted to the sulfate ester (8–10). When tamoxifen is given to female $\lambda\lambda\lambda\lambda\lambda$ transgenic rats hepatic DNA damage leads to an increase in the frequency of gene mutations at $lacI$ (11). In treated women there is evidence for an increased incidence of uterine endometrial (2,12) but not liver tumours (13). The presence of DNA damage in the target cells of tamoxifen treated women is controversial (14), and even if low levels of damage occurs (7), it is questionable if this is causally related to the increase in endometrial tumours (15,16). The role of $\alpha$-hydroxytamoxifen as a carcinogen or mutagen in rats or humans in vivo has not been established. In this study, the relationship between DNA damage resulting from the administration to rats of $\alpha$-hydroxytamoxifen and the subsequent development of liver lesions is investigated.

Materials and methods

Animals and treatments

$trans$ $\alpha$-Hydroxytamoxifen was prepared according to published procedures (17,18). Female 6-week-old Wistar/Han rats were obtained from Harlan Olac (Bicester, UK). 120 animals were housed in negative pressure isolators with a 12 h light/dark cycle at 19–22°C. Animals were dosed with $\alpha$-hydroxytamoxifen dissolved in tricaprylin, 40 animals at 0.103 mmol/kg and 40 animals at 0.0103 mmol/kg, intraperitoneally (40 and 4 mg/ml, respectively) daily for 5 days, while controls received tricaprylin only. At the end of the dosing period animals were divided into two groups. One group of 54 rats was subsequently given water supplemented with 0.1% (w/v) phenobarbital and 54 rats received no phenobarbital water supplement. At 24 h after the final dose and at 3 and 6 months, groups of four animals were killed for all treatments, leaving 10 rats/group. The study was terminated at 13 months. In a separate experiment, four female Wistar/Han rats were dosed with tamoxifen (0.103 mmol/kg, dissolved in tricaprylin), intraperitoneally, daily for 5 days while four controls received tricaprylin only. Animals were killed 24 h after the last dose.

For mutation assays, 10 female $\lambda\lambda\lambda\lambda\lambda$ transgenic rats (Big Blue rats, 6–8 weeks of age) homozygous for the $lacI$ gene (Stratagene, La Jolla, CA) were dosed orally with $\alpha$-hydroxytamoxifen dissolved in tricaprylin at 0.103 mmol/kg. In this assay, animals were dosed by gavage for 10 days. A control group of 10 animals received tricaprylin (1 ml/kg/day) for 10 days. For the $lacI$ mutation assay, all the animals were killed 46 days after the last dose. The diet was withdrawn 24 h prior to killing.

Tissue preparation

Livers were removed, weighed and a sample snap frozen in liquid nitrogen for DNA isolation. Sections of all major lobes of the liver and other organs were fixed in formal buffered saline for histological examination and in Carnoy’s fluid and ice-cold acetone for immunohistochemical studies.

Mutant frequency at $lacI$ gene in the liver

The $lacI$ mutation assay and the DNA sequence analysis of mutant $lacI$ genes was performed as described previously (11). Sequencing of the double-stranded DNA was carried out in both directions for 25 of the control-derived mutants and 23 of the $\alpha$-hydroxytamoxifen-derived mutants.

Abbreviations: BrdU, 5-bromodeoxyuridine; GST-P, placental form of glutathione S-transferase; PB, phenobarbital; PCNA, proliferating cell nuclear antigen.

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Isolation of hepatocytes

Hepatocytes were isolated from rats treated with α-hydroxytamoxifen, 0.103 mmol/kg intraperitoneally for 5 days or tricaprylin vehicle and killed on day 6. A two stage collagenase perfusion was used (19). This yields cell preparations consisting of >90% hepatocytes. Only preparations of >85% viability were used, as judged by Trypan blue exclusion and counting with a haemocytometer.

\[ \text{α-treatment dosing with} \]

\[ \text{Isolation of hepatocytes} \]

\[ \text{Liver pathology and GST-P foci with} \]

\[ \text{α} \]

\[ \text{fi} \]

\[ \text{control values. Rats given 0.103 mmol/kg} \]

\[ \text{554} \]

\[ \text{fi} \]

\[ \text{ANOVAR) with Dunnett} \]

\[ \text{(Minitab, PA). Difference between groups was tested using analysis of variance} \]

\[ \text{349 adducts/108 nucleotides while control hepatocytes showed} \]

\[ \text{Statistical analysis was carried out were carried out using Minitab version 10} \]

\[ \text{α} \]

\[ \text{Serotec, Oxford, UK) and 3,3'-diaminobenzidine/H}_2\text{O}_2 \text{substrate. Sections 108 nucleotides seen in rats given an equimolar dose of} \]

\[ \text{α-hydroxytamoxifen (Figure 1). Since} \]

\[ \text{GST-P positive foci. These foci increased in number and area} \]

\[ \text{Adducts were determined in DNA isolated from livers or hepatocytes using the} \]

\[ \text{23} \]

\[ \text{Post-labelling analysis} \]

Adducts were determined in DNA isolated from livers or hepatocytes using the \[2^{32}\text{P-post-labelling assay as described previously (20).} \]

\[ \text{Immunodetection of liver GST-P and cell proliferation by PCNA expression} \]

For the detection of GST-P foci, paraffin sections (5 μm) from acetonite-fixed livers were rehydrated and GST-P proteins were located immunohistochemically using an anti-GST-P polyclonal antibody (1:100 dilution) followed by an anti-rabbit alkaline phosphatase conjugated second antibody (1:50 dilution; Sigma). Bound antibody was detected using naphthol AS/BI phosphate and Fast Red TR as described previously (6). GST-P foci were defined as groups of five or more cells. Foci were counted on at least 1 cm\(^2\) of liver sections from animals at each time point. Areas of foci were calculated using an Analytical Measuring Systems VIDS V Imaging System (Synoptics, Cambridge, UK) and were expressed as number/cm\(^2\).

\[ \text{Statistical analysis was carried out were carried out using Minitab version 10} \]

\[ \text{α} \]

\[ \text{Forty-six days following oral dosing with} \]

\[ \text{α-hydroxytamoxifen at 0.103 or 0.0103 mmol/kg, hepatic DNA damage of about 700 adducts/10}^8 \text{ nucleotides. When Wistar rats were given areas were not signi} \]

\[ \text{α} \]

\[ \text{Forty-six days following oral dosing with} \]

\[ \text{α-hydroxytamoxifen, corresponding control groups. There was no difference in} \]

\[ \text{32P-post-labelling assay as described previously (20).} \]

\[ \text{Liver DNA damage caused by α-hydroxytamoxifen and tamoxifen in Wistar/Han rats} \]

After dosing rats with α-hydroxytamoxifen at 0.103 or 0.0103 mmol/kg for 5 days, total liver DNA damage, as determined by \[3^{2}\text{P-post-labelling 24 h after the last dose and at 3 months after cessation of dosing, is shown in Figure 1.} \]

\[ \text{Discussion} \]

This study shows that treatment of rats with α-hydroxytamoxifen for 5 days was sufficient to cause cumulative hepatic DNA damage, as measured by \[3^{2}\text{P-post-labelling, in the order of} \]

\[ \text{5340 adducts/10}^8 \text{nucleotides. When Wistar rats were given dietary tamoxifen for 3 months and then returned to a normal diet, hepatic DNA damage of about 700 adducts/10}^8 \text{nucleotides, determined by} \]

\[ \text{3^{2}\text{P-post-labelling, resulted in five out of} \]

\[ \text{treatment. There was no biliary proliferation in either of the} \]

\[ \text{Discussion} \]

This study shows that treatment of rats with α-hydroxytamoxifen for 5 days was sufficient to cause cumulative hepatic DNA damage, as measured by \[3^{2}\text{P-post-labelling, in the order of} \]

\[ \text{5340 adducts/10}^8 \text{nucleotides. When Wistar rats were given dietary tamoxifen for 3 months and then returned to a normal diet, hepatic DNA damage of about 700 adducts/10}^8 \text{nucleotides, determined by} \]

\[ \text{3^{2}\text{P-post-labelling, resulted in five out of} \]
Table I. Effects of α-hydroxytamoxifen treatment on the numbers and areas of GST-P foci

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GST-P foci</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (foci/cm²)</td>
<td>Area (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 months</td>
<td>6 months</td>
<td>13 months</td>
<td>13 months</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>Vehicle</td>
<td>0 (4)</td>
<td>1.2 ± 1.1 (4)</td>
<td>5.4 ± 1.9 (4)</td>
<td>0.05 ± 0.02 (9)</td>
</tr>
<tr>
<td></td>
<td>Vehicle + PB</td>
<td>0.17 ± 0.17 (4)</td>
<td>1.4 ± 0.7 (4)</td>
<td>7.7 ± 2.0 (6)</td>
<td>0.22 ± 0.10 (6)</td>
</tr>
<tr>
<td>α-Hydroxytamoxifen</td>
<td>0.0103 mmol/kg</td>
<td>ND b</td>
<td>2.2 ± 1.6 (3)</td>
<td>10.7 ± 2.6 (10)</td>
<td>0.08 ± 0.02 (10)</td>
</tr>
<tr>
<td></td>
<td>0.0103 mmol/kg + PB</td>
<td>ND b</td>
<td>3.6 ± 3.4 (4)</td>
<td>5.2 ± 1.9 (5)</td>
<td>0.12 ± 0.10 (5)</td>
</tr>
<tr>
<td></td>
<td>0.103 mmol/kg</td>
<td>14.5 ± 6.0 (4)</td>
<td>14.0 ± 6.9 (3)</td>
<td>24.1 ± 8.1 (7)*</td>
<td>0.64 ± 0.29 (7)</td>
</tr>
<tr>
<td></td>
<td>0.103 mmol/kg + PB</td>
<td>13.7 ± 3.8 (4)</td>
<td>22.1 ± 12.4 (4)</td>
<td>34.8 ± 7.1 (5)*</td>
<td>1.60 ± 0.54 (5)*</td>
</tr>
</tbody>
</table>

aResults represent the mean ± SD, the number of animals is shown in parentheses.
bNot determined.
*Significantly different from the corresponding controls at the 5% level by ANOVAR.

Fig. 1. DNA damage in the livers of rats given α-hydroxytamoxifen. α-Hydroxytamoxifen was dosed (0.103 or 0.0103 mmol/kg, intraperitoneally) to rats for 5 days and the animals killed on day 6, or 3 months later. Controls received tricaprylin vehicle. DNA was extracted from the liver and DNA damage determined by ³²P-post-labelling. The data shown are DNA adducts/10⁸ nucleotides (bars, SD) in animals treated with α-hydroxytamoxifen at 0.103 mmol/kg (□), 0.0103 mmol/kg (■) or vehicle dosed controls (○).

seven animals developing liver tumours at 12 months but only when promoted with phenobarbital (6). In contrast, no animal had liver tumours at this time without phenobarbital promotion. DNA damage of 2800 adducts/10⁸ nucleotides seen after 6 months continuous tamoxifen treatment, resulted in three out of five animals developing liver tumours in the absence of phenobarbital promotion (4). An investigation using female Sprague–Dawley rats found that daily treatment by gavage of 22.6 mg/kg tamoxifen for 12 months, resulted in a 100% incidence (24 out of 24 rats) of hepatocellular adenomas and carcinomas (5). Liver DNA adducts were not determined in this study.

In rats, an apparent causal relationship between DNA damage and liver tumours has been established. Dosing rats with tamoxifen analogues such as toremifene and droloxifene, compounds that cause little or no hepatic DNA damage, does not result in liver tumours (5,24,25). In tamoxifen treated rats, tumours only develop at the major site of DNA damage in the liver. A single dose of tamoxifen that leads to ~30 adducts/10⁸ nucleotides in the liver, as determined by ³²P-post-labelling

Table II. Number of plaques screened and mutations found at the lacI gene in rat liver

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plaques screened</th>
<th>Mutants</th>
<th>MF×10⁻⁵</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>339 391</td>
<td>10</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Rat 1</td>
<td>340 591</td>
<td>24</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>Rat 2</td>
<td>339 690</td>
<td>19</td>
<td>5.6</td>
<td>5.04 ± 1.7</td>
</tr>
<tr>
<td>Rat 3</td>
<td>325 266</td>
<td>20</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>Rat 4</td>
<td>335 490</td>
<td>12</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>α-Hydroxytamoxifen</td>
<td>311 025</td>
<td>19</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>Rat 1</td>
<td>314 709</td>
<td>33</td>
<td>10.5</td>
<td>9.48±3.3</td>
</tr>
<tr>
<td>Rat 2</td>
<td>313 349</td>
<td>27</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Rat 3</td>
<td>312 428</td>
<td>46</td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td>Rat 4</td>
<td>335 176</td>
<td>25</td>
<td>7.5</td>
<td></td>
</tr>
</tbody>
</table>

aDose: 0.103 mmol α-hydroxytamoxifen/kg by gavage for 10 days.
Fig. 3. The percentage of various mutation at the lacI gene in the livers of control or α-hydroxytamoxifen-treated (0.103 mmol/kg) homozygous lacI rats. The data shown are percentages (bars, SD) in the control (□) and tamoxifen-treated (●) animals.

(26), does not act as a tumour initiator even in the presence of phenobarbital promotion (27). The absence of liver tumours can be correlated with a low incidence of hepatic GST-P foci. In the present study, the area or number of such foci are only ~10% those seen in Wistar rats exposed to dietary tamoxifen that develop liver tumours (4).

Following the administration of tamoxifen to rats, about 0.1% of the dose is excreted via the bile as α-hydroxytamoxifen glucuronide (23). On this basis, a dose of 0.103 mmol/kg tamoxifen would be metabolized to the equivalent of 0.001 mmol/kg α-hydroxytamoxifen. The comparatively high doses of α-hydroxytamoxifen needed to get hepatic DNA damage in the present study suggests rapid inactivation occurs in the rat. Oral administration results in much lower levels of hepatic DNA damage suggesting inactivation probably associated with acidic pH values of the stomach. Although α-hydroxytamoxifen has been detected in the blood of patients given tamoxifen (28) in the present investigation, this compound was below the limit of detection in plasma 24 h after the last dose using HPLC with UV detection (29) (<100 ng/ml; I.N.H. White, unpublished).

α-Hydroxytamoxifen causes a significant increase in mutation frequency in the livers of female I/N lacI transgenic rats. There was no significant increase in G:C to T:A transversions, previously characterized as typical for tamoxifen induced mutations at lacI in these animals (11). Both the in vitro reaction of α-acetoxytamoxifen, a model ester of α-hydroxytamoxifen with DNA and the administration of tamoxifen to rats lead to the same major adduct, the trans form of α-(N-2-deoxyguanosinyl)tamoxifen (30,31). A number of other products are formed in the liver including (N-2-deoxyguanosinyl)-N-desmethyltamoxifen (31), but as judged by HPLC, the overall pattern of DNA adducts following the administration of α-hydroxytamoxifen is very similar to that seen after tamoxifen treatment of rats (18). Other metabolites, such as those formed by the peroxidasen activation of 4-hydroxytamoxifen, form adducts which co-elute with minor adducts in tamoxifen treated rats (20), but these adducts are more mutagenic than those derived from the in vitro reaction of α-acetoxytamoxifen, as shown using the lacI gene polymerase STOP assay (32).

Results show that the initiation of DNA damage by α-hydroxytamoxifen is not sufficient to lead to the development of hepatocellular carcinomas even with phenobarbital promotion. While qualitatively, the pattern of adducts formed in the liver following dosing with tamoxifen or α-hydroxytamoxifen are the same, the relative proportions of the different adducts differ. Not all of the DNA damage detected may be directly associated with the subsequent development of liver tumours. This has been established, for example, with a number of N-nitrosodialkylamine alkylating agents where most of the ring nitrogen atoms and the exocyclic oxygen atoms of guanine residues in DNA are targets (33), but all are not equivalent in terms of carcinogenic potential (34). Defining the relationship between the level of DNA damage and the length of time needed to allow promotion and progression of transformed cells will be of importance in understanding the mechanism of carcinogenicity.

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References

Short-term dosing of α-hydroxytamoxifen


