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Antiestrogens and the formation of DNA damage in rats: A comparison

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Abstract

Tamoxifen (TAM) has been used as an agent for the treatment and prevention of breast cancer. However, long-term treatment of TAM in women increases a risk of developing endometrial cancer. The secondary cancer may be due to the genotoxicity of TAM. To find safer alternatives, four selective estrogen receptor modulators (SERMs), 4-hydroxytamoxifen (4-OHTAM), toremifene (TOR), raloxifene (RAL) and ICI 182,780, were administered to rats with an equimolar dose of TAM [54 μ mol/kg (20 mg/kg)/day, p.o. for 7 days]. To evaluate the genotoxicity of each SERMs, the presence of bulky DNA adducts was determined by ³²P-postlabeling/polyacrylamide gel electrophoresis and ³²P-postlabeling/high performance liquid chromatography. The formation of 7,8-dihydro-8-oxodeoxyguanosine (8-oxodG) was analyzed as a marker of typical oxidative damage, using liquid chromatography electrospray tandem mass spectrometry. Among the SERMs, bulky DNA adducts were detected in the liver of rats treated with TAM; total amounts of TAM-DNA adducts was 26.1 adducts/10⁷ nucleotides. However, with a detection limit of ~2 adducts/10⁹ nucleotides, no bulky DNA adducts were observed with 4-OHTAM, TOR, RAL or ICI 182,780. In addition, no significant increase of hepatic 8-oxodG lesions was detected in rats treated with any of the antiestrogens. Therefore, TOR, RAL and ICI 182,780 are likely to be less genotoxic than TAM.

Introduction

Selective estrogen receptor modulators (SERMs) have been used as agents for the treatment and prevention of breast cancer (1). Tamoxifen (TAM, the structure in Fig. 1), the first clinically relevant SERM, has widely been used as a first-line endocrine therapy for breast cancer patients with estrogen receptor (ER) positive and is also a prophylactic agent for women at a high risk of this disease. Besides this significant benefit, long-term treatment of TAM in women increases the risk of developing endometrial cancer (2–5). The carcinogenic effects may be due to the estrogenic activity of TAM through the ER (6,7) and/or genotoxicity of TAM (reviewed by 8).

In rats treated with TAM, a high level of TAM-DNA adducts are produced in the liver (9–12) and initiate the development of hepatocellular carcinomas (13,14). TAM-DNA adducts are

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formed through *O*-sulfonation of α -hydroxylated TAM metabolites (8); α -(*N*²-deoxyguanosinyl)tamoxifen (dG-*N*²-TAM) and α -(*N*²-deoxyguanosinyl)-*N*-desmethyltamoxifen (dG-*N*²-*N*-desTAM) are detected as major hepatic DNA adducts using mass-spectroscopic and ³²P-postlabeling/HPLC analyses (12,15). There is a controversy regarding the detection of TAM-DNA adducts in the human tissues (16,17). TAM-DNA adducts were detected in endometrial tissues of breast cancer patients treated with TAM, using ³²P-postlabeling analysis (18) and accelerator mass spectrometry (19). Other groups did not detect TAM-DNA adducts in the endometrial tissues (20–22). A high frequency of *K-ras* mutations was recently observed in the endometrium of women treated with TAM (23); the mutational specificity was consistent with that occurred at the TAM-DNA adduct (24).

In addition, 4-hydroxytamoxifen (4-OHTAM), a principle TAM metabolite, may be activated metabolically to the quinone methide and quinone that can react with DNA and protein (Fig. 2A) (25–27). An *in vitro* activation of 4-hydroxytamoxifen (4-OHTAM) regenerated reactive oxygen species resulted in the increased formation of 7,8-dihydro-8-oxodeoxyguanosine (8-oxodG), an abundant mutagenic DNA lesion (28,29). Such oxidative DNA damage may also be involved in TAM-induced cancers.

Toremifene (TOR, Fig. 1), a chlorinated TAM derivative, has been used in breast cancer therapy. The clinical efficacy of TOR in treating advanced breast cancer was similar to that of TAM (30). Although TOR is metabolized similar to TAM (8), this drug is not a hepatocarcinogen in rats (14,31). There has not been any significant clinical report that TOR increases the incidence of endometrial cancer.

Raloxifene (RAL, Fig. 1) is currently used for osteoporosis, but has not yet been approved by the Food and Drug Administration (FDA) for breast cancer therapy. A recent chemoprevention trial showed that RAL significantly reduced the incidence of breast cancer in women at high risk of developing this disease (32). RAL has been shown to be metabolically activated to electrophilic and redox active quinoids that can react with protein including GST (Fig. 2B) (33). 8-OxodG may be produced through redox cycling between RAL catechols and *o*-quinones. These results suggest that electrophilic species of RAL may have the potential to damage macromolecules to cause the toxicity.

ICI 182,780 (Fig. 1) is a pure antiestrogen, which blocks the tropic action of estradiol in the rat uterus and is free from estrogen agonist activity (34). ICI 182,780 is approved for treatment of post-menopausal breast cancer patients who fail to respond to TAM therapy (35). Since the structure of ICI 182,780 resembles 17 β -estradiol, this drug may be similarly metabolized and could react with DNA. However, the contribution of reactive oxygen species generated from ICI 182,780 to genotoxicity has not been explored.

To determine the genotoxic potential of SERM, female rats were treated for a week with 4-OHTAM, TOR, RAL or ICI 182,780 and compared with TAM. The level of hepatic DNA adducts induced by each drug was examined using ³²P-postlabeling/polyacrylamide gel electrophoresis (PAGE) and ³²P-postlabeling/high performance liquid chromatography (HPLC) analyses. The formation of 8-oxodG was analyzed by liquid chromatography electrospray tandem mass spectrometry (LC/MS/MS) analysis.

Materials and Methods

Materials

TAM, TOR and RAL were purchased from LKT Laboratories, Inc. (St. Paul, MN). ICI 182,780 was obtained from TOCRIS (Ellisville, MO). 4-OHTAM, calf thymus DNA, micrococcal nuclease, and potato apyrase were purchased from Sigma-Aldrich (St. Louis, MO). Spleen

phosphodiesterase was obtained from Worthington Biochemical Corp. (Lakewood, NJ). 3'-Phosphatase-free T4 PNK and nuclease P1 were obtained from Roche Applied Science (Indianapolis, IN). *Trans*-forms (fr-1 and fr-2) and *cis*-forms (fr-3 and fr-4) of dG_{3'}-monophosphate-*N*²-tamoxifen (dG_{3'}-*N*²-TAM) were prepared as described previously (36). [γ -³²P]-ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Biosciences Corp. (Piscataway, NJ).

Animal study

Fisher 344 rats (female, 8 weeks-old) were purchased from Taconic (Germantown, NY). The use of animals was in compliance with the guidelines established by the NIH Office of Laboratory Animal Welfare. Animals were acclimated in temperature (22 ± 2 °C) and humidity (55 ± 5%) controlled rooms with a 12 hr light-dark cycle for at least 1 week prior to use. Regular laboratory chow and tap water were allowed *ad lib*. Rats were treated orally with TAM [20 mg (54 μmol)/kg/day] for 7 days. 4-OHTAM (21 mg/kg), TOR (22 mg/kg), RAL (26 mg/kg), or ICI 182,780 (33 mg/kg) was given to rats an equimolar dose of TAM (54 μmol/kg). Control rats were treated with an identical volume of corn oil. TAM-treated rats were euthanized by CO₂ asphyxiation at 5 hr after the final treatment, and open thoracotomy. All tissues were removed quickly, frozen, and stored at -80 °C until DNA extraction.

Digestion of DNA samples

The tissue DNA was extracted using a Qiagen DNA isolation kit (Valencia, CA) following the manufacturer's protocol. The concentration of DNA was determined by UV spectroscopy as 50 μg/mL = O.D._{260 nm} 1.0. DNA sample (2.5 μg) was enzymatically digested overnight at 37 °C in 100 μL of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM CaCl₂, using micrococcal nuclease (30 units) and spleen phosphodiesterase (0.15 unit) (37). The reaction mixture was incubated for another 1 h with nuclease P1 (1 unit). After the incubation, 150 μL of water was added. The reaction samples were then extracted twice with 200 μL of butanol. The butanol fractions were combined, back-extracted with 50 μL of distilled water, and evaporated to dryness.

³²P-Postlabeling/PAGE analysis

The DNA digests were incubated at 37 °C for 40 min with 10 μCi of [γ -³²P]-ATP and 3'-phosphatase-free T4 polynucleotide kinase (10 units), and then incubated for another 30 min with apyrase (50 milliunits), as described previously (37). Known amounts (0.076 pmol, 0.0076 pmol, 0.00076 pmol or 0.000076 pmol) of dG-*N*²-TAM-modified oligodeoxynucleotide were mixed with 2.5 μg of calf thymus DNA (7600 pmol) and served as a standard (1 adduct/10⁵ nucleotides, 1 adduct/10⁶ nucleotides, 1 adduct/10⁷ nucleotides, or 1 adduct/10⁸ nucleotides). A part of the ³²P-labeled sample was electrophoresed for 4–5 h on a non-denaturing 30% polyacrylamide gel (35 × 42 × 0.04 cm) with 1400–1600 V/20–40 mA. The position of ³²P-labeled adducts was established by β-phosphorimager analysis (Molecular Dynamics Inc.). To quantify the level of ³²P-labeled products, integrated values were measured using a β-phosphorimager and compared with the standards. The detection limit for 5 μg DNA was approximately 7 adducts/10⁹ nucleotides.

³²P-Postlabeling/HPLC analysis

When the ³²P-labeled products were developed on the gel as described above, the bands of ³²P-labeled products were cut from the gel and put into 2 mL of distilled water for overnight at the room temperature. The ³²P-labeled products extracted from the gel were evaporated to dryness. Recovery of ³²P-labeled products was ~95%. The ³²P-labeled products were solved in 20 μL of distilled water and subjected to a Hypersil BDS C₁₈ analytical column (0.46 × 25 cm, 5 μm, Shandon), eluted at a flow rate of 1.0 mL/min with a linear gradient of 0.2 M

ammonium formate and 20 mM H₃PO₄, pH 4.0, containing 20 to 30 % acetonitrile for 40 min, 30 to 50 % acetonitrile for 5 min, followed by an isocratic condition of 50 % acetonitrile for 15 min (37). The radioactivity was monitored using a radioisotope detector (Berthold LB506 C-1, ICON Scientific Inc.) linked to a Waters 990 HPLC instrument. Known amounts (0.076-0.000076 pmol) of dG-N²-TAM-modified oligodeoxynucleotide were mixed with 2.5 µg of calf thymus DNA (7600 pmol) and served as a standard. As described previously (37), the amount of TAM-DNA adducts detected increased linearly depending on the amounts of oligodeoxynucleotide used. The detection limit of this assay was ~2 adducts/10⁹ nucleotides for 5 µg DNA.

Determination of 8-oxodG by LC/MS/MS analysis

The hepatic DNA was extracted by a modified method of Wang et al. (38). Liver tissue (100 mg) was homogenated at 4 °C in 0.2 mL of lysis solution A (320 mM sucrose, 5 mM MgCl₂, 10 mM Tris, 0.1 mM desferrioxamine pH 7.5, 1% Triton X-100) by disposable pellet pestle (Kimble/Kontes, Vineland, NJ). After tissue grinding, the homogenate was subjected to centrifugation at 10,000 xg for 20 sec at 4 °C. One mL of lysis solution A was added to the pellet and agitated by vortexing. This process was repeated twice. After centrifugation, 200 µL of solution B (10 mM Tris, 5 mM EDTA-Na₂, 0.15 mM desferrioxamine, pH 8.0) and 20 µL of 10 % SDS were added to the pellet. The mixture was vortexed for 10 sec and incubated at 37 °C for 10 min for suspension of pellet and allow for lysis of the nuclear membrane completely. After addition of 2.7 µg of RNase T₁ and 10 µg RNase A in a buffer (10 mM Tris, 1 mM EDTA, 2.5 mM desferrioxamine, pH 7.4), the samples were incubated at 50 °C for 15 min. Following incubation, 10 µL of Qiagen proteinase K solvent was added and the samples were incubated for 1 h at 37 °C. After addition of 0.3 mL of NaI solution (7.6 M NaI, 40 mM Tris, 20 mM EDTA-Na₂, 0.3 mM desferrioxamine, pH 8.0), DNA was precipitated by addition of 500 µL of 2-propanol. The DNA lump was fished out and washed twice with 40 % 2-propanol. Finally the recovered DNA was dried and dissolved in 500 µL of distilled water. The concentration of DNA was determined by UV spectroscopy.

The level of 8-oxodG was determined using liquid chromatography electro spray tandem mass spectrometry (LC/MS/MS) (HPLC: a Shimadzu LC-10ADvp pump and SIL-10AD auto injector; MS/MS: Waters-Micromass Quattro Ultima TMPt Triple Quadrupole mass spectrometer). To quantify 8-oxodG accurately, ¹⁵N₅-8-oxodG was prepared from ¹⁵N₅-dG those all five Ns in guanine base are replaced by ¹⁵N (Cambridge Isotope Lab), following a method developed previously (39) and used as an internal standard. The DNA (50 µg) was mixed with ¹⁵N₅-8-oxodG (500 pg), digested at 37 °C for 3 h with nucleaseP1 (4 unit) in 114 µL of buffer mixture (100 µL of 30 mM sodium acetate buffer containing 10 mM 2-mercaptoethanol, pH 5.3; 5 µL of 20 mM ZnSO₄; 5 µL of ¹⁵N₅-8-oxodG solution (5 ng/mL); 4 µL of nuclease P1 solution (1 unit/µL); 3 units of alkaline phosphatase). After incubation, 20 µL of 0.5 M Tris-HCl, pH 8.5 was added and incubated for 3 h. The enzymes were precipitated using methanol and the supernatant containing the nucleoside was evaporated and reconstituted with 100 µL of water.

The LC column was eluted over a gradient that began at a ratio of 2 % methanol to 98 % water and was changed to 40 % methanol over a period of 40 min, changed to 80 % methanol from 40 min to 45 min, and finally returned to the original starting conditions, 2:98, for the remaining 15 min. The total run time was 60 minutes. Sample injection volumes of 50 µL each were separated on a Shim-pack FC-ODS column (150× 4.6 mm) and eluted at a flow rate of 0.4 mL/min. Mass spectral analyses were carried out in positive ion mode with nitrogen as the nebulizing gas. The ion source temperature was 130 °C, the desolvation gas temperature was 380 °C, and the cone voltage was operated at a constant 35 V. Nitrogen gas was also used as the desolvation gas (700 L/h) and cone gas (35 L/h) and argon was used as the collision gas at

a collision cell pressure of 1.5×10^{-3} mBar. Positive ions were acquired in MRM mode. The MRM transitions monitored were as follows: $^{15}\text{N}_5$ -8-oxodG (m/z 288.8 $\rightarrow$ 172.8) and 8-oxodG (m/z 283.8 $\rightarrow$ 167.8), respectively. The amount of 8-oxodG in DNA sample was determined by comparing with $^{15}\text{N}_5$ -8-oxodG. The detection limit was approximately 0.1 adducts in 10^6 bases using 50 μg of DNA. The amount of dG in the DNA digest was monitored by Shimadzu SPD-10A UV-Visible detector prior to MS/MS analysis. The level of 8-oxodG lesions was estimated by the following equation: the level of 8-oxodG lesion = (amount of 8-oxodG)/[(amount of dG) $\times$ 4].

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (S.D.). Two sided student's *t*-test was used to evaluate the difference. Values of $p \leq 0.05$ were considered statistically significant from control group.

Results

To investigate the genotoxicity of each SERM, rats were treated orally for 7 days with TAM, 4-OHTAM, TOR, RAL, or ICI 182,780 at an equimolar dose of TAM (54 $\mu\text{mol/kg/day}$). The level of hepatic DNA adduct was determined using ^{32}P -postlabeling/PAGE. A high amount of TAM-DNA adducts (total 26.1 ± 9.5 adducts/ 10^7 nucleotides) were detected in all the rats treated with TAM (Fig. 3). To determine the TAM-DNA adducts, the adduct was recovered from the gel and subjected to HPLC inlined with a radioisotope detector. Compared with standards of TAM-DNA adducts (Fig. 4A), *trans*-forms (fr-2) of dG $_{3'p}$ - N^2 -TAM and dG $_{3'p}$ - N^2 -*N*-desTAM were major adducts at the level of 12.1 ± 4.6 adducts/ 10^7 nucleotides and 11.0 ± 4.4 adducts/ 10^7 nucleotides, respectively (Fig. 4B). Using both ^{32}P -postlabeling/PAGE and ^{32}P -postlabeling/HPLC, no DNA adducts were detected in liver of rats treated with 4-OHTAM, TOR, RAL, or ICI 182,780 (Figs. 3 and 4).

The level of hepatic 8-oxodG lesions induced by SERM was determined using LC/MS/MS analysis and an internal standard, $^{15}\text{N}_5$ -8-oxodG. Analysis of $^{15}\text{N}_5$ -8-oxodG and 8-oxodG was achieved by monitoring the MS/MS transitions corresponding to the loss of deoxyribose from $^{15}\text{N}_5$ -8-oxodG (m/z 288.8 $\rightarrow$ 172.8) and 8-oxodG (m/z 283.8 $\rightarrow$ 167.8), respectively (Fig. 5). The amount of 8-oxodG in DNA samples was determined by comparing with $^{15}\text{N}_5$ -8-oxodG. The detection limit for 50 μg DNA sample was approximately 0.1 adduct/ 10^6 nucleotides. The level of 8-oxodG in the liver of rats treated with TAM and 4-OHTAM were 2.17 adducts/ 10^6 nucleotides and 1.97 adducts/ 10^6 nucleotides, respectively, those were slightly higher than that (1.78 adducts/ 10^6 nucleotides) of rats treated with vehicle; however, no significant difference was observed (Table 1). The levels of 8-oxodG induced by TOR, RAL, and ICI 182,780 were similar to that of the control.

Discussions

To determine the genotoxic potential, we have selected 4-OHTAM, TOR, RAL and ICI 182,780 that are currently approved by FDA for breast cancer therapy or under the clinical trial and compared their genotoxic potential with that of TAM. Rats were treated with each antiestrogen at an equimolar dose of TAM (54 $\mu\text{mol/kg/day}$). The level of bulky and oxidative DNA damage in the liver was determined using ^{32}P -postlabeling analyses and LC/MS/MS, respectively.

TAM induced a high level of TAM-DNA adducts in rat liver; that major adducts were confirmed as a *trans*-isomer (fr-2) of dG- N^2 -TAM and dG- N^2 -*N*-desTAM (Fig. 4), as observed previously by several research groups including us (8). Interestingly, the formation of hepatic TAM-DNA adducts was not observed with a *trans*-isomer of 4-OHTAM, a principle TAM

metabolite having antiestrogenic activity. Our results are consistent with that observed with rats treated orally with a mixture of *trans* and *cis*-isomers (~1:1) of 4-OHTAM (21 mg/kg/day for 7 days) (40), indicating that 4-OHTAM is not involved in the formation of TAM-DNA adducts. Based on the results obtained in vitro experiments (28,29), 4-OHTAM is suspected to oxidize cellular DNA through the redox cycling between the hydroxyl and quinone forms (Fig. 2A). However, significant increase of 8-oxodG adducts were not observed in the liver of rats treated with either TAM or 4-OHTAM. A similar result was reported using a different strain in rat treated with TAM; however, no detailed information, including the dose, duration and route of TAM treatment were described in this study (41). These results support that 4-OHTAM-derived quinone methide and/or *o*-quinone (25–27) are not significantly involved in the formation of oxidative and bulky TAM-DNA adducts in vivo.

Since TOR is a chlorinated TAM, TOR may be metabolized similarly to TAM (8). However, treatment of TOR to rats did not promote bulky DNA adducts in the liver. Our results are consistent with the fact that TOR promoted two orders of magnitude lower levels of DNA adducts in rat liver compared to TAM (14,31). We found previously that TOR is α -hydroxylated 6-fold higher by cytochrome 3A4 than TAM (42), but unlike α -OHTAM, α -OHTOR is not a substrate for hydroxysteroid sulfotransferase and the reactivity of α -acetoxyTOR, a model activated from TOR, with DNA was much less than that of α -acetoxyTAM (43). In addition, like TAM, TOR did not promote oxidative DNA damage. Therefore, TOR may not have genotoxic potential. In fact, no mutagenic events were detected in liver of λ /lacI rats treated with TOR (44). Only one out of 21 patients treated with TOR had a *K-ras* mutation whereas a high frequency of *K-ras* mutation was detected in endometrial tissues of patients treated with TAM (23). The replacement of TAM by TOR may minimize the development of endometrial cancer observed in women treated with TAM.

In this study, we couldn't detect significant bulky and oxidative DNA adducts induced by RAL or ICI 182,780 in the rat livers. Actually, no increased incidence of endometrial cancer was observed in women treated with RAL (45,46). Since RAL and ICI 182,780 have only a minimal estrogenic activity (47,48), use of both compounds may have more clinical benefit for breast cancer therapy and prevention. However, since RAL has two hydroxyl moieties in the structure, the hydroxylated compound can rapidly conjugate through phase II metabolism and get excreted. As a result, higher doses may be required to be administered in human to obtain an efficacy equivalent to that with TAM (49). ICI 182,780 is highly lipophilic and does not ionize at physical condition (50). This may make it difficult for human to reach adequate bioavailability by oral administration.

Only TAM generated significant amount of DNA adduct. If the mutagenic TAM-DNA adducts are not efficiently repaired, TAM-DNA adducts could cumulate for extended periods in the targeted tissues, leading to the development of cancers (51). TOR, RAL, and ICI 182,780 were observed to be less genotoxic than TAM. Although the effects of these antiestrogens on ER pathways should be evaluated, TOR, RAL, and ICI 182,780 may be safer alternatives for breast cancer therapy and chemoprevention.

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Abbreviations

SERM

selective estrogen receptor modulator

ER	estrogen receptor
TAM	tamoxifen
α-OHTAM	α -hydroxytamoxifen
4-OHTAM	4-hydroxytamoxifen
TOR	toremifene
RAL	raloxifene
dG-N^2-TAM	α -(N^2 -deoxyguanosinyl)tamoxifen
dG-N^2-N-desTAM	α -(N^2 -deoxyguanosinyl)- N -desmethyltamoxifen
8-oxodG	7,8-dihydro-8-oxodeoxyguansine
PAGE	polyacrylamide gel electrophoresis
HPLC	high performance liquid chromatography
LC/MS/MS	liquid chromatography electrospray tandem mass spectrometry

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Figure 2.
A proposed mechanism of forming TAM-DNA adduct derived from 4-OHTAM.

Figure 3.

³²P-Postlabeling/PAGE analysis for determination of DNA adducts in rat liver. Three each rats were treated orally with either TAM, TOR, RAL, ICI 182,780, 4-OHTAM or vehicle at a molecular equivalent dose of TAM [54 μ mol (20 mg)/kg/day] for 7 days. The hepatic DNA was used for analysis of DNA adducts using ³²P-postlabeling/PAGE, as described in the Experimental Procedure. Standard (Stn.) is a 2.5 μ g calf thymus DNA (0.76×10^7 fmol dNs) containing a known amount (3.8 fmol dNs) of a trans-form (fr-2) of dG-*N*²-TAM-modified oligomer, representing 5 adduct/ 10^7 dNs.

Figure 4.

³²P-Postlabeling/HPLC analysis for determination of DNA adducts in rat liver. The hepatic DNA was used for analysis of DNA adducts using ³²P-postlabeling/HPLC, as described in the Experimental Procedure; Standards (A), TAM (B), Control (C), TOR (D), and RAL (E).

Figure 5.

LC/MS/MS analysis of 8-oxodG.

The DNA (50 μg) was mixed with $^{15}\text{N}_5\text{-8-oxodG}$ (500 pg), digested with nuclease P1, and alkaline phosphatase in a buffer. The resulting nucleosides were analyzed using LC/MS/MS, as described in the Experimental Procedure. Quantative analysis of $^{15}\text{N}_5\text{-8-oxodG}$ and 8-oxodG was achieved by monitoring the MS/MS transitions corresponding to the loss of deoxyribose from $^{15}\text{N}_5\text{-8-oxodG}$ (m/z 288.8 $\rightarrow$ 172.8) (A) and 8-oxodG (m/z 283.8 $\rightarrow$ 167.8) (B), respectively. The amount of 8-oxodG in DNAsamples was determined by comparing with $^{15}\text{N}_5\text{-8-oxodG}$.

Table 1

Level of hepatic 8-oxodG adducts in rats treated with antiestrogens.

	8-oxodG (adducts/10 ⁶ dNs)	
Control	1.78 ± 0.51	(100 %)
TAM	2.17 ± 0.36	(122 %)
4-OHTAM	1.97 ± 0.14	(111 %)
TOR	1.82 ± 0.33	(102 %)
RAL	1.92 ± 0.35	(108 %)
ICI 182,780	1.80 ± 0.39	(101 %)

Data are expressed as mean values ± S.D. from three rats.