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Differences in Metabolite-mediated Toxicity of Tamoxifen in Rodents vs. Humans Elucidated with DNA/microsomes Electro-optical Arrays and Nanoreactors

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Abstract

Tamoxifen, a therapeutic and chemopreventive breast cancer drug, was chosen as a model compound because of acknowledged species specific toxicity differences. Emerging approaches utilizing electro-optical arrays and nanoreactors based on DNA/microsomes films were used to compare metabolite-mediated toxicity differences of tamoxifen in rodents vs. humans. “Hits” triggered by liver enzyme metabolism were first provided by arrays utilizing a DNA damage endpoint. The arrays feature thin-film spots containing electrochemiluminescent (ECL) ruthenium polymer ([Ru(bpy)₂PVP₁₀]²⁺; PVP = polyvinylpyridine), DNA, and liver microsomes. When DNA damage resulted from reactions with tamoxifen metabolites, it was detected by an increase in light from the oxidation of the damaged DNA by the ECL metallopolymer. The slope of ECL generation vs. enzyme reaction time correlated with the rate of DNA damage. An approximate 2-fold ECL turnover rate was observed for spots with rat liver microsomes compared to human liver microsomes. These results were supported by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of reaction products using nanoreactors featuring analogous films on silica nanoparticles, allowing direct measurement of relative formation rate for α -(N²-deoxyguanosinyl)tamoxifen. We observed 2–5 fold more rapid formation rates for three major metabolites, i.e. α -hydroxytamoxifen, 4-hydroxytamoxifen, and tamoxifen N-oxide catalyzed by rat liver microsomes compared to human liver microsomes. Comparable formation rates were observed for N-desmethyl tamoxifen with rat and human liver microsomes. A better detoxifying capacity for human liver microsomes than rat liver microsomes was confirmed utilizing glucuronyltransferase in microsomes together with UDP-glucuronic acid. Taken together, lower genotoxicity and higher detoxication rates presented by human liver microsomes correlate with the lower risk of tamoxifen in causing liver carcinoma in humans, provided the glucuronidation pathway is active.

Introduction

Tamoxifen (TAM)¹ was the first cancer chemopreventive drug approved by the U.S. Food and Drug Administration (FDA) and has been the gold standard for the treatment of estrogen-receptor-positive breast cancer over the past three decades (1,2). Tamoxifen has also been found to reduce the overall risk of invasive breast cancer by 49% in ‘at risk’ women (3). More

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¹Abbreviations: TAM, tamoxifen; α -OHTAM, α -hydroxytamoxifen; 4-OHTAM, 4-hydroxytamoxifen; N-desTAM, N-desmethyltamoxifen; TAM N-oxide, tamoxifen N-oxide; UGT, UDP-Glucuronyltransferase; UDPGA, UDP-glucuronic acid; CapLC, capillary liquid chromatography; MS/MS, tandem mass spectroscopy; MRM, multiple reaction monitoring; QCM, quartz crystal microbalance.

than twenty years after introduction into the clinic, tamoxifen was found to produce liver tumors in rats in a conventional 2-year carcinogenicity bioassay (4). Later, reports revealed a slight but statistically significant increase in risk of endometrial cancer in tamoxifen treated patients (5,6), as well as in healthy women enrolled in chemopreventive trials (3). The question as to whether tamoxifen forms adducts in endometrial DNA in treated women has been controversial. Some investigations failed to detect tamoxifen-DNA adducts in the endometrium of patients using ^{32}P -post-labelling with TLC or HPLC and mass spectrometry (7–9), whereas others detected low levels of tamoxifen-DNA adducts in uterine tissue of women treated with tamoxifen for varying lengths of time utilizing ^{32}P -post-labeling/HPLC and accelerator mass spectrometry (10–13). Still, with the level of DNA adducts detected, whether these adducts are related to the development of endometrial cancer in women treated with tamoxifen remains uncertain.

The metabolism of tamoxifen in humans qualitatively resembles that of rodents, which involves oxidation and bioconjugation pathways (Scheme 1). Tamoxifen is bioactivated by cytochrome P450 enzymes yielding demethylation and hydroxylation products, and by flavin-containing monooxygenase producing an N-oxygenated product. Major oxidative metabolites of tamoxifen found in human plasma include α -hydroxytamoxifen (α -OHTAM), N-desmethyl tamoxifen (N-desTAM), tamoxifen N-oxide (TAM N-oxide), 4-hydroxytamoxifen (4-OHTAM), and several other secondary metabolites (14,15). The α -hydroxylated metabolites play major roles in toxicity, since they can be bioconjugated by hydroxysteroid sulfotransferase to give sulfate esters as putative reactive intermediates (16,17). These intermediates react with the exocyclic amino groups of guanines (the major reaction) and adenines (a minor reaction) in DNA, forming two *trans* and two *cis* diastereoisomers of tamoxifen-nucleobase adducts (18). Another postulated genotoxic pathway involving 4-OHTAM quinone methide intermediates does not seem to be involved in causing DNA damage *in vivo* (19). In terms of excretion and detoxification of tamoxifen, glucuronidation plays a key part and can deactivate parent drug or conjugate with α -hydroxylated metabolites competing with its further activation (20–22).

It is acknowledged that tamoxifen is genotoxic in rat liver, as indicated by the formation of DNA adducts of its metabolites (24). However, the risk of liver DNA damage in women taking this drug is fairly low (2). The fact that tamoxifen is not considered a human liver carcinogen can be explained by the metabolism of tamoxifen in humans compared to that of rodents, which include (i) the low activity of P450 3A enzymes towards α -hydroxylation of tamoxifen in humans (25), (ii) the resulting low level of sulfate ester formation because of the low activity of human hydroxysteroid sulfotransferase (17), and (iii) a more efficient glucuronidation process leading to detoxified conjugates in humans (26). Taking the normal therapeutic dose of tamoxifen into account, it has been estimated that the overall hepatic safety factor of tamoxifen in women is ~60,000 larger than in rodents (2).

We recently developed predictive *in vitro* assays that screen reactive metabolite with a DNA damage end point (27–30). This technology employs thin films containing metabolic enzymes and DNA, in which test compounds are first metabolized into potentially reactive metabolites that are then trapped by DNA in the film as nucleobase adducts. High concentrations of DNA in the film insure good probability of adduct formation from reactive metabolites. Relative formation rates of DNA adducts are first detected by an electro-optical (electrochemiluminescent, ECL) array. Significant toxic “hits” identified by significantly high rates of ECL generation vs. reaction time can be followed up by direct measure of formation rates and structural identification of the DNA adducts by capillary LC- tandem mass spectroscopy (MS/MS) analysis of reaction products using DNA/microsomes nanoreactors. This two-tiered *in vitro* genotoxicity screening approach provides the potential for high-

throughput reactive metabolites screening providing chemical reactivity and structural information (28).

Herein, we demonstrate for the first time that this drug toxicity screening approach is capable of elucidating species specific metabolic and toxicity differences. This was done by comparative studies of tamoxifen using the first tier ECL arrays with spots comprised of ds-DNA, the electrochemiluminescent ruthenium polymer ($[\text{Ru}(\text{bpy})_2\text{PVP}_{10}]^{2+}$) (RuPVP), and rat or human liver microsomes. The second tier analysis features 500 nm diameter silica microspheres (nanoreactors) coated with analogous DNA/microsomes films used to form reactive metabolites and DNA adducts detected by LC-MS/MS. We also included UDP-glucuronic acid (UDPGA) in the arrays and nanoreactors to assess the glucuronyl conjugation for the first time, enabling observation of the superior detoxification effects of tamoxifen in human liver microsomes. These studies showed that rat liver microsomes produces DNA adducts of tamoxifen at much faster rates than human liver microsomes, and that human liver microsomes provides more rapid detoxication via an efficient glucuronidation pathway.

Experimental Procedures

Chemicals and Materials

The electrochemiluminescent ruthenium metallopolymer was synthesized and characterized following an established protocol (31). α -OHTAM, N-desTAM, and TAM N-oxide were obtained from Toronto Research Chemicals (Ontario, Canada). Silica microspheres were from Polysciences Inc. (Warrington, PA. 500 nm ($\pm 10\%$) diameter, approximately 10% solids, $d = 1.96 \text{ g cm}^{-3}$). All other chemicals were from Sigma-Aldrich. Rat liver microsomes (pooled, Fischer 344), human liver microsomes (pooled, female) and rat liver cytosol (pooled, Sprague Dawley) were supplied by BD biosciences (Woburn, MA).

Film Fabrication

Array spots containing rat or human liver microsomes were assembled on a 1×1 in. pyrolytic graphite (PG) chip using established protocols (27,28). The following solutions were used for film assembly: 2 mg mL^{-1} calf thymus DNA (10 mM, Tris pH 7.4, 50 mM NaCl), 2.5 mg mL^{-1} RuPVP 88 (88% $\text{H}_2\text{O}/12\%$ ethanol), 2.5 mg mL^{-1} RuPVP 50 (50% $\text{H}_2\text{O}/50\%$ ethanol), and 20 mg mL^{-1} total protein rat or human liver microsomes (in 250 mM sucrose 10 mM Tris pH 7.4), with a final film construction of $\text{DNA}/(\text{RuPVP88}/\text{DNA})_2/(\text{RuPVP50}/\text{DNA}/\text{microsomes})/(\text{RuPVP88}/\text{DNA}/\text{microsomes})_2$. Similar film assembly on 500 nm-diameter silica beads was done as previously reported (28) with a few modifications. Briefly, 800 μL of poly(diallyldimethylammonium chloride) (PDDA) (2 mg mL^{-1} , 50 mM NaCl) was added to 1 mL (1.96 g mL^{-1}) of silica beads and was allowed a 15-min assembly. The suspension was centrifuged for 1 min at 8000 rpm and rinsed with water to remove loosely bound polyions. The beads were then dispersed in 800 μL of ds-DNA solution (0.5 mg/mL) allowing a 15-min assembly, which was followed by rinsing. Similar steps were followed for polystyrene sulfonate (PSS) (2 mg mL^{-1} , 50 mM NaCl) or microsomes adsorption. After fabrication, the films had the following architecture: PDDA/PSS/PDDA/microsomes for metabolite identification or $(\text{PDDA}/\text{DNA})_2/\text{PDDA}/\text{microsomes}$ for DNA adduct determination. The modified beads were dispersed in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) to a final volume of 1 mL and stored near 0 °C till use.

Metabolic Activation and Detoxication

(1) ECL Array—A 50 mM MES buffer (pH 6.0) solution containing tamoxifen (25 μM) and NADPH generating system (10 mM glucose 6-phosphate, 4 units of glucose-6-phosphate dehydrogenase, 10 mM MgCl_2 , 0.80 mM NADP^+) was incubated at 37 °C for 10 min before exposing to array spots. A 3 mM UDPGA was present in the above solution when detoxication

pathways were examined. Reaction was initiated by treating the array spots with the aforementioned solution and stopped by rinsing the solution off the arrays with water. The array is placed in an open-top electrochemical cell in a dark box with a CCD camera mounted on top to measure the ECL light emission and the ECL image was collected and processed following using previously described methods (27a).

(2) Nanoreactors—Metabolic enzyme reactions were started by mixing the DNA/microsomes coated silica beads (150 μL) with equal volumes of 50 mM MES buffer (pH 6.0) with tamoxifen (25 μM) and NADPH generating system. Reactions were stopped by adding 150 μL cold acetonitrile followed by immediate centrifugation. For determination of tamoxifen metabolites, the supernatant was collected and filtered through Centricon filters with cut-off mass of 3000 Da (Amicon, Beverly, MA) and analyzed by capillary LC (CapLC)-MS/MS. For determination of DNA adducts, DNA was hydrolyzed by formic acid using a previously described method (27b) that releases damaged nucleobases as well as some free bases. Briefly, the supernatant was discarded, and a mixture of the beads and 300 μL formic acid (>98%, Fluka) was heated to effect acid hydrolysis (60 min, $\sim 140^\circ\text{C}$) in a glass acid hydrolysis tube (Kontes). Afterwards, acid was removed by a vacufuge concentrator (Eppendorf) at 60°C , and the hydrolysate was dissolved in 200 μL 10 mM ammonium acetate buffer (pH 4.5), and was filtered through Centricon filters with cut-off mass of 3000 Da, followed by CapLC-MS/MS analysis.

(3) Preparation of α -OHTAM glucuronide and α -(N^2 -deoxyguanosinyl) tamoxifen Standards— α -OHTAM glucuronide was prepared by incubating 100 μM of α -OHTAM with human liver microsomes (2 mg of protein), 3 mM UDPGA and 8 mM MgCl_2 in 0.5 ml of 50 mM potassium phosphate (pH 7.0) at 37°C for 1 h. α -(N^2 -deoxyguanosinyl) tamoxifen standard was prepared from previously reported methods with modification (32). Briefly, calf thymus DNA (60 μg) was incubated at 37°C for 1 h with cytosol (2 mg of protein), 100 μM α -OHTAM, 200 μM PAPS, and 100 μM EDTA in 0.5 ml of 50 mM MES buffer (pH 6.0). The DNA was recovered using the ethanol precipitation and hydrolyzed by aforementioned formic acid hydrolysis. All samples were filtered through Centricon filters prior to LC-MS analysis.

CapLC-MS/MS Analysis

(1) CapLC—The capillary LC system (Waters, Milford, MA) was equipped with column switching, which allows the selective capture of nucleobase adducts in a trapping column, while the unmodified nucleobases were directed to waste. The trapping column (Atlantis dC18, 23.5 mm, 0.18 mm i.d., 5 μm particle size) and analytical column (Atlantis dC18, 150 mm, 300 μm i.d., 5 μm particle size) were from Waters (Milford, MA). A 10 μL portion of the sample was loaded into the trapping column at a flow rate of $4.25 \mu\text{L min}^{-1}$ and flushed with 60:40 ammonium acetate buffer/methanol (v/v) at a flow rate of $10 \mu\text{L min}^{-1}$ for 4 min before switching to in-line position. Elution onto the analytical column was achieved at a flow rate of $4.25 \mu\text{L min}^{-1}$ with the following gradient: (A, 10 mM ammoniumacetate buffer, pH 4.5 with 0.1% formic acid; B, methanol with 0.1% formic acid) 5min, 40% B; 10 min, 40–75% B; 20min 75–84% B; 10min, 84–100% B; 5min 100% B; 10min 100–40% B. A photodiode array detector was used to monitor the wavelength 210–300nm throughout the analysis.

(2) On-line CapLC-MS/MS—A Micromass Quattro II mass spectrometer (Beverly, MA) with electrospray source was operated in the positive ion mode. Samples were analyzed with a source block temperature of 70°C and a desolvation temperature of 70°C . Nitrogen was used as the desolvation (250 L/h) and nebulizing (90 L/h) gas. Argon was used as the collision gas, at a collision cell pressure of 5×10^{-3} mBar. Tandem MS were acquired in the daughter (DAU) and multiple reaction monitoring (MRM) mode to study the fragmentation pattern and relative

quantity of tamoxifen-DNA adducts (cone voltage 15 V, collision energy 9eV, dwell time 0.3 s, span 0.02 Da, and interchannel delay 0.03 s).

Results

Array Film Characterization

Quartz crystal microbalance (QCM) analysis was employed to monitor the quality of film growth and the amount of microsomes loaded on the film. QCM frequency shifts obtained during films growth were nearly linear (Supporting information, Figure 1), suggesting regular film growth with reproducible layers of DNA and microsomes. The estimated amounts of DNA and microsomes, and the average thickness of the films were summarized in Table 1.

ECL Array Responses

(1) Cytochrome P450 Mediated Tamoxifen Activation—We previously demonstrated that ECL arrays featuring DNA/microsomes films can detect metabolite-related DNA damage (27–30). The slope of the relative ECL generation vs. reaction time correlates with the rate of nucleobase adduct formation, i.e. the rate of DNA damage, measured directly by LC-MS/MS (27–30). The Ru^{II}PVP polymer catalyst in the films is oxidized electrochemically to Ru^{III}PVP at ~1.1V in the detection step, and subsequently oxidizes guanines on DNA, yielding an electronically excited Ru^{II}*PVP that emits visible ECL light (31). The formation of reactive metabolite-DNA adducts partially disrupts the DNA double helix, enhancing access of the catalytic Ru^{III}-centers to guanines leading to increased oxidation rates and increased ECL light emission for damaged DNA (31).

The ECL array image in Fig. 1A portrays *in vitro* metabolic activation when RuPVP/DNA/microsomes array spots are exposed to 25 μ M tamoxifen and NADPH for various times in seconds. The ECL intensity increase reflects the increase in relative amount of DNA adducts of tamoxifen after metabolic activation (Fig. 1A, labeled as TAM/R and TAM/H). Images of control incubations with the NADPH regenerating system only (images not shown) resemble that of tamoxifen only (Fig. 1A, control). The normalized ECL increase can be obtained from relative ECL intensity increase divided by the estimated amount of microsomes in the film (from QCM), as shown in Fig. 1B. The relative turnover rates as % ECL increase (min mg microsomes)⁻¹ obtained from the slopes of normalized ECL increase vs time were 78($\pm$ 10) for rat and 45($\pm$ 8) for human liver microsomes arrays, respectively. These values were shown to be significantly different by a t-test at the 95% confidence level (Supporting Information, Table S1). The 1.7-fold larger ECL turnover rate for rat liver microsomes compared to human liver microsomes reflects a faster DNA adduct formation rate for rat liver microsomes. The negligible ECL turnover rates in control reactions indicate the need for combined tamoxifen and NADPH exposure to achieve bioactivation.

(2) UDP-Glucuronyltransferase (UGT) Mediated Detoxication of Tamoxifen—

Glucuronidation is a major elimination and detoxication route of tamoxifen and its metabolites. A glucuronic acid moiety can be bioconjugated to either parent drug at N,N-dimethylamino alkyl side chain or *O*-glucuronidated to α -OHTAM or 4-OHTAM to reduce their availability for subsequent DNA damage (20–22). Particularly, human liver microsomes exhibited higher glucuronidation activity toward α -OHTAM, reportedly 50 times more rapid than rat liver microsomes (26). In order to scrutinize the inter-species differences in detoxication effects of UGT on tamoxifen metabolism using ECL arrays, 3 mM of cofactor UDPGA was included in the enzymatic reactions. A significant decrease in ECL intensity was found for rat liver microsomes spots (Fig. 1A, UGT/R), but an even greater ECL intensity decrease occurred for human liver microsomes (Fig. 1A, UGT/H). Decreases in ECL intensities for both films suggest slower DNA adduct formation rates when bioconjugation catalyzed by UGT is active.

Normalized ECL data in Figure 1B reveals a ~50% decrease with UDPGA compared to that without UDPGA for rat liver microsomes films which were shown to be significantly different by t-tests at at 95% confidence levels (Supporting Information, Table S1), whereas a nearly zero turnover rate not significantly different from controls at 95% confidence levels was found for films of human liver microsomes with UDPGA. This disparity in normalized turnover rates, i.e., normalized slopes of ECL intensity vs. reaction time obtained from figure 1B, reveals the different relative efficiencies of UGT bioconjugation reactions in competition with DNA damage in rat and human liver microsomes. These data demonstrate a superior detoxication profile towards tamoxifen in human liver microsomes films.

Metabolites and DNA Adducts Analysis by LC-MS/MS

(1) Metabolites Analysis—Microsomes/polyion nanoreactor particles were reacted with tamoxifen and the NADPH regenerating system, and formation of several metabolites after 1 min reaction was revealed in representative chromatograms (Fig. 2A, red, rat liver microsomes and green, human liver microsomes). Four major metabolites represented in Scheme 1, i.e., α -OHTAM, 4-OHTAM, N-des TAM, and TAM N-oxide, were identified by matching the retention times with available authentic standards (Fig. 2A, blue). Metabolites of tamoxifen catalyzed by human liver microsomes qualitatively resemble that of rat liver microsomes, however obvious disparities are present in the formation rates of different metabolites, as summarized in Table 2. Rat liver microsomes feature a faster metabolism of tamoxifen, giving 2~5-fold higher rates for three of the metabolites compared to that of human liver microsomes, except for N-des TAM. N-desmethylation, being the predominant biotransformation for tamoxifen, presents comparable turnover rates for both rat and human liver microsomes. Particularly, a 2.6-fold higher formation rate of α -OHTAM mediated by rat liver microsomes compared to human liver microsomes predicts a higher potency in DNA adducts formation, which was observed as a higher turnover rate in ECL response (*vide supra*).

(2) DNA Adduct Analysis—Covalent binding of tamoxifen metabolites to DNA was confirmed using similar enzymatic reactions catalyzed by nanoreactors with DNA/microsomes films followed by formic acid hydrolysis to release DNA adducts as bases and deoxynucleosides (27b). Better signal to noise was afforded by measuring LC-MS/MS of adducted deoxynucleosides. Tamoxifen-guanine base adduct with a mass transition from 261 to 185 was present as a minor product (Supporting information, Figure S3). The same mass transitions at the similar retention times were obtained using the authentic synthesized α -(N²-deoxyguanosinyl)tamoxifen and tamoxifen-guanine base adduct standards in order to confirm the formation of tamoxifen adducted deoxynucleoside and free base, and their release from the damaged DNA (Supporting information, Figure S2). Doubly charged α -(N²-deoxyguanosinyl)tamoxifen ($m/z=319$) was thus identified as the major adduct with a similar fragmentation pattern as in earlier studies (33) (Fig. 2C, inset). Fig. 2C illustrates the daughter ions of parent ion, $m/z=319$, in which $m/z=261$ and $m/z=185$ are proposed by previous reports to be the doubly charged tamoxifen-guanine moiety and the tamoxifen moiety, respectively (34). A series of MRM chromatograms with mass transition from 319 to 261 can be attained with an increase in peak area vs. reaction time (Fig. 2B), indicating the increase of the relative amount of α -(N²-deoxyguanosinyl)tamoxifen in the damaged DNA with reaction time. Similarly, the normalized turnover rates of α -(N²-deoxyguanosinyl)tamoxifen were derived from the slopes of peak area/amount of microsomes in the film vs. reaction time for both rat and human liver microsomes reactions (Fig. 2B, inset). A 1.9-fold higher normalized turnover rate (Table 2) for rat liver microsomes vs. human liver microsomes correlates well with 1.7-fold higher ECL turnover rate. The results confirm the relative higher formation rate of tamoxifen-DNA adduct in rat liver microsomes film, which is in accordance with our observation in ECL arrays, indicating a faster DNA damage effect triggered by rat liver microsomes.

(3) UGT Mediated Detoxication of Tamoxifen—In order to confirm the bioconjugation route of tamoxifen metabolites observed in the ECL arrays, 3 mM UDPGA was included in enzymatic reactions. In the presence of UGT and cofactor, formation of α -OHTAM glucuronide was detected by monitoring the mass transition from m/z 564 (protonated α -OHTAM glucuronide) to m/z 370 (tamoxifen moiety) at 17.9 min as shown in Fig. 2D, as reported by previous studies (14). The identity of α -OHTAM glucuronide was further confirmed the correspondence of the retention time with that of the authentic α -OHTAM glucuronide standard (data not shown). The generation of α -OHTAM glucuronide confirms the previous results from ECL responses that bioconjugation reactions metabolized by UGT in human liver microsomes did occur and plays a detoxication role in eliminating the reactive metabolite(s). This mass transition was not detected for the rat liver microsomes reaction at the same condition, suggesting better efficiency of UGT or more favorable competition with DNA damage in human compared to rat liver microsomes. When increased levels as low as 30 μ M of UDPGA were used in the reaction, DNA adducts level fell below the detection limit for human liver microsomes reactions, implying that glucuronidation can compete with further possible toxication to eliminate DNA adduct formation (26).

Discussion

Results above demonstrate that the two-tiered drug toxicity screening approach involving electro-optical array analysis with follow up LC-MS/MS detection of metabolites and DNA-adducts is applicable to address complex specific metabolism and species toxicity differences. In this approach, toxic “hits” signaling different toxicology profiles were initially provided by ECL arrays, and then DNA/microsomes nanoreactors and LC-MS/MS analysis were used to obtain structural details and formation rates for the metabolites and DNA adducts for both rat and human liver microsomes. In this study, ECL arrays suggested a faster bioactivation of tamoxifen toward DNA damage for rodents than for humans. Follow up investigations using nanoreactor/LC-MS validated the array responses with relative DNA adduct formation rates and structural determinations, as well as formation rates of metabolites. Formation rates of reactive intermediates and DNA adducts measured directly by LC-MS/MS (cf. Figure 2a,b) correlated very well with the rates of ECL signal increase (Figure 1). In comparison to traditional solution methods for metabolites and DNA adducts identification, DNA/enzyme nanoreactors provide an environment with high local concentration of enzymes and DNA to rapidly generate metabolic products and DNA adducts in sufficient amounts for LC-MS analysis. Using this approach, significant amounts of target metabolites and their DNA adducts can be detected in several minutes.

Furthermore, insights into the detoxifying capacity of human and rat liver microsomes were gained through ECL intensity decrease by utilizing glucuronyltransferase together with its cofactor. The spots containing human liver microsomes presented a greater decrease in ECL output suggested a more efficient detoxication process by human liver microsomes than rat liver microsomes (Fig. 1B). One of the major detoxication pathways, *O*-glucuronidation of α -OHTAM, was later proved by LC-MS/MS analysis using microsomes nanoreactors.

In the ECL arrays, α -OHTAM formed in the film (confirmed by LC-MS), catalyzed by microsomal enzymes, can play an important part in DNA adduct formation at pH 6.0, since α -OHTAM can directly react with DNA in acidic medium without further metabolic activations (18), as illustrated in Scheme 1. DNA adduct formation mediated by 4-OHTAM quinone methide upon microsomal activation might also play a role here, as suggested by earlier studies (35). A nearly 2-fold higher normalized ECL turnover rate of rat liver microsomes compared to that of human liver microsomes reveals the faster DNA adducts formation rate in rat liver microsomes films, indicating faster bioactivation of tamoxifen catalyzed by rat liver microsomes.

Follow-up LC-MS studies confirmed the formation of reactive metabolites generated in the array films. The relative formation rates of tamoxifen metabolites obtained in two kinds of microsomal films confirm earlier studies using solution reactions (25,36). Small discrepancies in details may arise from differences in enzyme source, methodology, and incubation time. Higher turnover rates of metabolites in our study compared with solution reactions may be due to the large reactive surface area of nanoreactors and high local concentrations enzyme of generated metabolites (28,37). A 1.9-fold higher formation rate of α -(N²-deoxyguanosinyl) tamoxifen mediated by rat liver microsomes compared to human liver microsomes obtained from LC-MS/MS studies correlates well with the ECL array responses.

The variations in ECL array output before and after introducing the cofactor of UGT suggests that glucuronidation competes with DNA damage (22). A nearly zero turnover rate of tamoxifen-DNA adduct observed for human liver microsomes spots implies a very efficient bioconjugation process. This was further verified by the formation of α -OHTAM glucuronide using LC-MS/MS. The failure of detection of similar product in rat liver microsomes film may due to the other elimination pathway mediated by UGT, such as quaternary ammonium-linked glucuronidation of tamoxifen (20). These studies show that rat liver microsomes produce DNA adducts at a much faster rate than human liver microsomes, and that human liver microsomes provides more rapid detoxication via an efficient glucuronidation pathway, which could partly explain the low risk of tamoxifen in causing human liver carcinoma. However, the relative risk could be elevated for individuals whose the glucuronidation pathway is inactive.

In summary, the two-tiered array and nanoreactor/LC-MS toxicity screening approach was able to easily and rapidly establish significant differences in bioactivation and detoxication mediated by microsomal enzymes of rodents and humans, offering mechanistic clarification of the underlying species specific toxicological mechanisms. Findings using this approach confirmed a more toxic profile of tamoxifen in rat liver than in human liver. The usefulness of this approach for prediction of genotoxicity of a new chemical/drug candidate in early screening is prefigured by this study. Chemical information obtained in this way can be integrated with bioassay results to make decisions along the development pipeline, to direct follow-up bioassays, or to assist in risk/benefit assessment. However, the present results do not shed light on the risk of human endometrial cancer. Clearly, global toxicity predictions should rely on a comprehensive evaluation of collections assays to unveil issues that could be found much later in clinical trials and further work in required in this area.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Reconstructed array data for tamoxifen (TAM): (A) ECL from spots of RuPVP/DNA/rat liver microsomes (labeled TAM/R) or RuPVP/DNA/human liver microsomes (labeled TAM/H) exposed to 25 μ M tamoxifen using enzymatic NADPH regeneration for denoted time (in s). Control is an identical array exposed to 25 μ M tamoxifen. Detoxication pathway was examined at the presence of 3 mM UDP-glucuronic acid in enzymatic reaction. Spots with rat liver microsomes and human liver microsomes films were labeled as UGT/rat and UGT/human, respectively; (B) A normalized ECL increase [% ECL increase (min-mg microsomes)⁻¹] for rat liver microsomes (black), human liver microsomes (red), rat liver microsomes with 3 mM glucuronic acid (blue), human liver microsomes with 3 mM glucuronic acid (orange), control incubations with 25 μ M tamoxifen only (purple) and control with the NADPH regenerating system only (green). Each data represent mean $\pm$ SD from 12–16 spots from 3–4 trials.

Figure 2.

(A) LC chromatogram in blue represents commercial standards. a, α -OHTAM, 10 μ M. b, 4-OHTAM, 10 μ M. c, N-des TAM, 10 μ M. d, TAM, 10 μ M. e, TAM N-oxide, 30 μ M. LC chromatograms in red and green ($\times 3$) represent 1 min reaction using nanoparticles featuring rat and human liver microsomes, respectively, together with 25 μ M of tamoxifen and NADPH regenerating system. Chromatograms display at a wavelength of 236nm. (B) MRM chromatograms monitoring α -(N²-deoxyguanosinyl)tamoxifen formation using rat liver microsomes films with mass transition from 319 to 261. Chromatogram 1 obtained from control reaction with 25 μ M tamoxifen only. Chromatograms 2, 3 and 4 represent reactions for 1, 2 and 3 min, respectively. The inset is a normalized peak area vs. time, in which integrated peak areas of MRM chromatogram are normalized based on estimated amount of microsomes in the films, i.e. peak area/wt. rat liver microsomes (red) and human liver microsomes (blue). (C) Daughter ions of 319, with fragmentation pattern illustrated in the inset. (D) MRM chromatogram (564 $\rightarrow$ 370) representing the formation of α -OHTAM O-glucuronide by human liver microsomes after enzymatic reaction using nanoreactors for 1 min.

Scheme 1.

Toxication and detoxication pathways of tamoxifen in humans (2,18,23).

Table 1QCM Characteristics of DNA/Microsomes Films (data represent mean $\pm$ SD from three trials).

Film composition	Average Film Weight ($\mu\text{g cm}^{-2}$)			Average Film Thickness (nm)
	RuPVP	DNA	Microsomes	
DNA/Rat Liver Microsomes	3.3 $\pm$ 2.0	1.8 $\pm$ 0.4	4.8 $\pm$ 1.0	40 $\pm$ 8
DNA/Human Liver Microsomes	3.2 $\pm$ 0.3	2.1 $\pm$ 0.2	3.2 $\pm$ 0.3	25 $\pm$ 2

Initial Formation Rates ^a of Tamoxifen Metabolites and Relative DNA Adduct Turnover Rates ^b using Nanoreactors with Rat Liver Microsomes and Human Liver Microsomes Films and LC-MS/MS (mean ±SD from 5 trials).

Table 2

microsomes	α -OHTAM	4-OHTAM	N-des TAM	TAM N-oxide	DNA adduct turnover
Rat liver	0.091±0.020	0.63±0.17	1.3±0.4	0.047±0.008	0.36±0.06
Human liver	0.035±0.03	0.12±0.05	1.1±0.2	0.019±0.005	0.19±0.03

^a in nmol (min-mg microsomes)^{−1} using integrated peak areas from 210–300nm by photo diode array detector.

^b Integrated areas of MRM chromatogram vs. amounts of protein in films. area/ng microsomes.