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Human cyt P450 mediated metabolic toxicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) evaluated using electrochemiluminescent arrays†

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Abstract

Electrochemiluminescent (ECL) arrays containing polymer ([Ru(bpy)₂(PVP)₁₀]²⁺, PVP = polyvinylpyridine), DNA, and selected enzymes were employed to elucidate cytochrome (cyt) P450 dependent metabolism of the tobacco specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Bioactivated NNK metabolites formed upon H₂O₂-enzymatic activation were captured as DNA adducts and detected simultaneously from 36 spot arrays by capturing and quantifying emitted ECL with an overhead CCD camera. Increased ECL emission was dependent on NNK exposure time. Of the enzymes tested, the activity toward NNK bioactivation was cyt P450 1A2 > 2E1 > 1B1 ≈ chloroperoxidase (CPO) > myoglobin (Mb) in accordance with reported *in vivo* studies. Cyt P450/polyion films were also immobilized on 500 nm diameter silica nanospheres for product analysis by LC-MS. Analysis of the nanosphere film reaction media provided ECL array validation and quantitation of the bioactivated NNK hydrolysis product 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) confirming production of reactive metabolites in the films. Chemical screening in this fashion allows rapid clarification of enzymes responsible for genotoxic activation as well as offering insight into cyt P450-related toxicity and mechanisms.

Introduction

Metabolic and toxicity testing of chemicals and chemical mixtures designed for pharmaceutical, industrial, or personal use is a major undertaking because of the numbers of compounds, assay times, instrumental costs, and sometimes poor correlation of *in vitro* data to *in vivo* response.^{1,2} For compounds designed for human ingestion, like drugs and food additives, knowledge of the metabolic enzymes involved is paramount.³ Often, it is through the metabolic process that toxicity is imparted to an otherwise benign chemical compound.⁴ This process is termed “bioactivation” and must be discovered before a compound is released to the public.^{4,5} Undiscovered toxic metabolites have in part lead to highly publicized pharmaceutical side reactions that can eventually lead to large scale recalls costing huge monetary sums, and sometimes unwarranted mortality.^{5,6}

†Electronic supplementary information (ESI) available: Additional experimental details and Fig. S1–S3.

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From a non-pharmaceutical standpoint, diseases have been hypothesized to originate from exposure to ubiquitous compounds in the everyday environment, including those compounds in the food supply,⁷ drinking water,⁸ and air.⁹ However, a significant level of controversy exists in detailing what is actually toxic upon human consumption or exposure.^{10–12} Certainly, some of this controversy stems from genetic heterogeneity that predisposes certain individuals or populations to xenobiotic induced diseases based on slight differences altering the delicate balance between metabolism and DNA repair pathways.^{13,14} The enzymes predominately responsible for the primary metabolism of xenobiotics are the monooxygenase cytochrome (cyt) P450s present in all human tissues.⁵ Therefore, it is necessary to rapidly and affordably elucidate the P450 isoforms responsible for xenobiotic metabolism of chemical compounds in order to help alleviate any existing toxicity controversy and rapidly elucidate pathways of toxic metabolite formation.

We have previously demonstrated a high-throughput array-based metabolism assay designed to show enzyme–substrate specificity involving reactive metabolite detection *via* a DNA damage endpoint.^{15–17} Here, multiple spots constructed layer by layer (LbL) featuring of a DNA-reactive ruthenium polyvinylpyridine (RuPVP) electrochemiluminescent (ECL) generating polymer¹⁸ along with DNA and cyt P450 isozymes of interest are immobilized on a large 1 in² graphite block. The substrate reaction occurs after peroxide activation of the cyt P450s facilitating ferryl-oxo radical formation¹⁹ to produce identical products *vis-à-vis* the natural NADPH–reductase activation cycle.²⁰ After completion of the reaction, the electrode block is transferred into a dark box equipped with an overhead CCD camera that can visualize the ECL generated from the interaction of the RuPVP and DNA from each spot simultaneously upon the application of an external potential. ECL increase is proportional to the amount of reactive metabolite induced DNA damage.¹⁵ Higher catalytic turnovers and ECL outputs are due to the ease of ruthenium access to guanine bases in DNA slightly distorted by adduct formation of other bases with the metabolites.¹⁶ The relative increase in DNA damage is proportional to the amount of DNA-reactive metabolite generated and related to enzyme–substrate specificity. Validation and quantification of the sensor response can be achieved employing synergistic LC-MS methods where similar layered films can be immobilized on 500 nm silica nanoparticles (nanoreactors).^{15,17,21} Upon exposure to similar reaction conditions, reactive metabolites and metabolite–DNA adducts can be identified and their rates of formation compared to the increase of ECL response *vs.* time.

Cigarettes offer an excellent example of a complex ubiquitous chemical mixture as a source of etiologic cancer agents toward several different tissues.^{10,11,22,23} Over 4800 compounds exist in cigarette smoke, the most carcinogenic of which are the groups of polycyclic aromatic hydrocarbons (PAHs)^{24,25} and nitrosamines (*N*-nitroso compounds).²⁶ The toxicity of these compounds once ingested comes from their bioactivation by metabolic enzymes, primarily cyt P450s, to highly reactive forms.²⁷ In this fashion, PAHs are oxidized by cyt P450 family 1 enzymes to ultimate carcinogens *via* a number of routes.^{15,26,28,29} *N*-Nitroso compounds are predominately hydroxylated at the α -methylene or methyl positions next to the nitroso moiety, resulting in an unstable species that enters into a variety of reactive forms that can potentially adduct DNA.³⁰ A simplified bioactivation pathway is summarized in Scheme 1 using the *N*-nitroso ketone, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), as an example.

NNK is important due to its predominant presence in cigarettes, making it a potent *tobacco specific* carcinogen.³⁰ Furthermore, NNK is of primary interest in this family of tobacco specific nitrosamines due to its increased levels of toxicity compared to related analogues present in tobacco.³⁰ Methods to determine enzymatic specificity for the substrates in question have often employed solution based microsomal³¹ or reconstituted purified enzyme

systems³² that can offer the desired information, but are also time consuming and costly owing to the instrumentation and substrate and/or enzyme amounts needed. Herein we demonstrate the utility of ECL arrays to elucidate the specificity of different cyt P450s, and to identify the metabolites of NNK using a selection of metabolic liver and lung cyt P450s along with control enzymes with peroxidase activity.

Experimental section

Materials

Calf thymus double stranded DNA, type I, 41.9% G/C (DNA), chloroperoxidase (CPO), myoglobin (Mb), poly(diallyldimethylammonium chloride) (PDDA, a polycation), and poly(styrenesulfonate) (PSS, a polyanion) were purchased from Sigma. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) were obtained from Toronto research chemicals (North York, ON, Canada). Cytochrome (cyt) P450 enzymes 1A2 (MW 52 000),³³ 2E1 (MW 52 000),³⁴ and 1B1 (MW 52 000)³⁵ were expressed from DH5α *Escherichia coli* containing the proper cDNA, and were isolated and purified according to the referenced procedures. The concentration of the expressed P450s was assayed by the Omura and Sato method.³⁶ The activity of the purchased CPO was assayed by a *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) colorimetric assay (Fig. S1, ESI†).³⁷ The ECL polymer [Ru(bpy)₂(PVP)₁₀](ClO₄)₂ (RuPVP) was prepared following established literature protocols.^{18,38,39} All other chemicals were reagent grade.

ECL array fabrication

DNA/RuPVP/enzyme films for ECL arrays (6 × 6 spots) were constructed on a conducting basal plane pyrolytic graphite (PG) block (Advanced Ceramics, 2.5 cm² × 1 cm) by the LbL electrostatic assembly method similar to that described previously.^{15,19,40} The adsorbed layers were rinsed with water between adsorption steps to remove weakly adsorbed material. Film architectures expressed as adsorbed layer order for ECL arrays were DNA/(RuPVP/DNA)₄/(enzyme/DNA)₂ denoted as DNA/RuPVP/enzyme (where enzyme = cyt P450 1A2, 2E1, or 1B1, CPO or Mb). The concentrations of solutions used were as follows: 2 mg mL⁻¹ DNA in 10 mM Tris buffer with 50 mM NaCl, pH 7.1, 1 mg mL⁻¹ RuPVP (88% H₂O–12% ethanol), 1 mg mL⁻¹ RuPVP (50% H₂O–50% ethanol), myoglobin (3 mg mL⁻¹, 10 mM acetate buffer, pH 4.5), cyt P450 1A2, 1B1, and 2E1 (1 mg mL⁻¹, 50 mM potassium phosphate buffer, pH 7.0), CPO (1 mg mL⁻¹, 10 mM acetate buffer, pH 4.5). Each spot had a final area of 0.04 cm².

Incubation of ECL array spots with procarcinogen

Safety note: NNK and its metabolites are suspected human carcinogens. All procedures were done while wearing gloves, and under closed hoods. Film spots were exposed to 1 μL of 1 mM NNK and 1 mM H₂O₂ in 50 mM acetate buffer, pH 5.5 for different times at 37 °C. Upon completion of the substrate exposure, the array was rinsed in water to stop the reaction. Control array spots were incubated either in 1 mM NNK or 1 mM H₂O₂ under the same conditions.

Electrochemiluminescence (ECL)

The ECL detection method is same as that described elsewhere.¹⁵ Upon reaction completion and rinsing, the array electrode block was placed in a 100 mL beaker filled to ~50 mL with 10 mM sodium acetate buffer + 0.15 M NaCl, pH 5.5, and was placed in the gel documentation dark box (SynGene ChemiGeniusQ, Fredrick, MD) for light acquisition by the CCD camera (QI Cam (Q-Imaging), 12-bit (0–4095 greyscales), 10 000 electron linear full well capacity, 35% QE at 600 nm). ECL was produced upon application of +1.25 V (CH

Instruments 1232, Austin, TX) vs. Ag/AgCl for 20 s, and a signal was acquired in pixel binning high sensitivity mode. Data analysis and quantification were done using GeneSnap and GeneTools software provided by SynGene (Cambridge, UK, 2005). Faux color was added to the b/w CCD image using Irfanview (Ver. 4.20, Irfan Skiljan, Vienna, 1996–2008) and the included Adobe8F filters followed by .tif file and additional color enhancements using Photoshop CS software. Added color is defined by user preferences, and is not absolute.

Nanoreactor fabrication

Films were formed on hydroxylated SiO₂ (Polysciences, Inc., Warrington, PA, 500 nm dia.) nanoparticles using a previously established protocol.²¹ SiO₂/PDDA/PSS/enzyme [enzyme = cyt P450 1A2/2E1, or CPO or Mb] films were incubated in stirred solutions containing 1 mM of each NNK and H₂O₂ in 50 mM acetate buffer, pH 5.5 for 10 min at 37 °C. The reaction was stopped *via* centrifugation, and the supernatant was analyzed using capillary LC-MS to identify the major 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) hydrolyzed metabolite. HPB quantification was performed after construction of a calibration plot using commercially available HPB.

Capillary LC-MS

A trapping column (Atlantis, dC18, 23.5 mm, 0.18 mm, I.D. 5 µm particle size) and the analytical column (Atlantis, dC18, 150 mm, 300 µm, I.D. 5 µm particle size) were from Waters (Milford, MA). The capillary LC was equipped with a photo diode array detector (scanning wavelengths from 200–450 nm). A 10 µL sample injection for each analysis was loaded into the trapping column at a flow rate of 4.25 µL min⁻¹ and washed with water for 1 min at 10 µL min⁻¹. The trapped metabolite and substrate were then back-flushed from the trapping column onto the analytical column at 4.25 µL min⁻¹ using the following elution gradient: 5 min 10% B, 5 min 10% to 30% B, 30 min 30% B, 5 min 30% to 10% B, 5 min 10% B (A, 10 mM acetate buffer, pH 5.5; B, methanol). Electrospray ionization mass spectrometry (ESI-MS) employed a Micro-mass Quattro II (Beverly, MA) operated in the positive ion mode (ESI⁺). The following conditions were used: cone voltage = 15 V, collision energy = 20 eV and collision gas (Ar) pressure = 5 × 10⁻³ mbar. The metabolites were analyzed by total ion scan (TIC).

Results

Fig. 1 shows a reconstructed array demonstrating the ECL response from the DNA/RuPVP/enzyme films upon NNK–H₂O₂ exposure for the denoted times (seconds). The ECL is generated upon application of +1.25 V vs. Ag/AgCl, which oxidizes the Ru^{II} centers in the polymer to Ru^{III}. The oxidized Ru^{III} centers interact with the guanines in the DNA films to eventually produce an excited Ru^{II*} state that emits ECL upon relaxation to the original Ru^{II} form. The ECL increases concomitant with NNK reaction time compared to its initial baseline (*i.e.* no damage, 0 s) due to the enzymatic bioactivation of NNK producing reactive electrophilic species that diffuse through the films to form helix distorting DNA adducts on nucleophilic DNA. This distortion allows the closer approach of an electrode-oxidized Ru^{III} center to exposed DNA bases in the film, and a kinetically faster reaction producing increased amounts of ECL.¹⁸ In this fashion, the sensor will detect any adduct that results in double helix distortion, not just isolated (*i.e.* N7 guanine) positional isomers.^{17a} Similar voltammetric guanine detection has been described by Thorp *et al.* employing soluble Ru(bpy)^{2+/3+}.^{16,41}

Array reconstruction in this manner allows for a clear qualitative assessment of the enzymes that are active in the NNK reaction. The important aspect to consider when using the array

to discern the enzymes that are more active toward a particular tested substrate is the *change* in ECL, and that from the analytical spots is compared to the respective zero second exposure spots as an internal standard. In this fashion, compensation for any inter-experimental fluctuation in light output is achieved.¹⁵ The sensor has the ability to discriminate enzymes active toward a particular substrate *vs.* the ones that exhibit more sluggish reaction rates. From the figure, it is clear that both cyt P450 1A2 and 2E1 films exhibit the most ECL change over the exposure time range studied; therefore, these two enzymes are more active and specific toward NNK bioactivation whereas the other P450 isoform, 1B1, and the model oxidases, CPO and Mb, demonstrate no significant ECL increase, which equates to muted NNK specificity. Mb in fact showed absolutely no ECL flux across the studied time range, which is consistent with our earlier work concerning the lack of specificity of this enzyme for *N*-nitroso compounds.¹⁷ Controls for these experiments have been described previously^{15,17} and consisted of both an unreacted array (*i.e.* enzyme spots exposed to no NNK or H₂O₂) as well as an array exposed to an individual member of the damage cocktail (*i.e.* only NNK or H₂O₂). The ECL response under these control conditions does not change appreciably, denoting that the enzymes must be activated by H₂O₂, and no other aspects of the reaction or experimental setup are accountable for the ECL flux (data not shown).

Fig. 2 is the quantitative representation of Fig. 1 raw data results where the percent (%) ECL change *vs.* NNK–H₂O₂ reaction time is plotted for each enzyme film. The percent ECL change over reaction time was measured relative to each enzyme 0 s exposure spot. Initial slopes of the data plotted in Fig. 2 are indicative of enzyme-substrate turnover, as validated by LC-MS experiments.^{21b} Again, cyt P450 1A2 was the most active of the tested enzymes toward NNK bioactivation into reactive electrophilic intermediates. Cyt P450 2E1 showed intermediate activity toward NNK, while cyt P450 1B1 and CPO showed negligible activity in the studied time period. The results showing that cyt P450 1A2 and 2E1 are the most active toward NNK activation are consistent with previously published data showing that these enzymes play a significant role in the metabolism of this xenobiotic, which is discussed further below. NNK is also known to be metabolized in the lungs and respiratory tract organs and cyt P450 1B1 is an extrahepatic isoform found in those locales. The lack of ECL signal, however, suggests that this enzyme plays a very small role in any NNK activation *in vivo*. As stated previously, Mb demonstrated an ECL% increase slope approaching 0 (error bars $\sim \pm 5\%$); therefore, we did not involve that data in further statistical analysis. In addition, square wave voltammetry¹⁹ was performed employing single electrodes with identical DNA/RuPVP/enzyme layers to verify the ECL response. Indeed, cyt P450 1A2 showed a similar increase in the voltammetric response (higher peak current), while cyt P450 1B1, CPO, and Mb films showed very little change over the studied reaction period (Fig. S2, ESI†).

Table 1 couples the ECL data and the enzyme surface coverage amounts from each film to summarize relative enzyme turnover values for NNK metabolism. The enzyme surface coverage (Γ) for each enzyme film was obtained from quartz crystal microbalance (QCM) studies (Table S1, ESI†). Accounting for Γ allows for an accurate view of the actual enzyme activity. Consistent with Fig. 1 and 2, cyt P450 1A2 indeed shows the highest NNK turnover response. T-tests at the 95% confidence level elucidated that cyt P450 1A2 exhibited the largest statistically significant NNK turnover value, followed by cyt P450 2E1. Similar tests showed that there was an insignificant difference between cyt P450 1B1 and CPO toward NNK bioactivation despite differences in raw array ECL percentage increases. This is due to the G correction factor accounting for the increased amount of P450 1B1 *vs.* CPO present in each DNA/RuPVP spot.

In order to couple the ECL sensor with an absolute quantitative output, LC-MS methods were employed to study the metabolic output by immobilizing similar films on 500 nm dia. silica nanospheres (nanoreactors). Fig. 3 shows LC chromatograms and mass spectra monitoring HPB production from cyt P450 1A2, cyt P450 2E1, and CPO nanoreactor films after 10 min NNK-H₂O₂ exposure. HPB is a hydrolyzed NNK metabolite that results from the conversion of enzyme produced α -hydroxylated NNK into reactive intermediates that are attacked by the nucleophilic aqueous solvent forming an alcohol (Scheme 1). Therefore, HPB presence indicates that reactive NNK metabolites are being generated enzymatically. Fig. 3a shows that the production of HPB is clearly evident from the nanoreactors containing cyt P450 1A2 and 2E1, while it is absent upon inclusion of the CPO, consistent with the ECL array data. Cyt 1B1 films exhibited similar responses to those of CPO. HPB from these films is most likely produced at levels lower than the limits of UV detection. Fig. 3b and c demonstrates the MS spectra from the TIC chromatogram for the two different peaks in Fig. 3a, which exhibit m/z consistent with newly produced HPB (Fig. 3b) and unreacted NNK (Fig. 3c), respectively. Qualitatively, the cyt P450 1A2 nanoreactor HPB peak area in Fig. 3a approximately doubles that from cyt P450 2E1 films, and calibration curve quantification verifies this observation (Fig. S3, ESI†).

We previously utilized the nanoreactor platform to detect and elucidate the DNA adducts that were being formed in the immobilized films. We showed that cyt P450 2E1 produced reactive NNK metabolites that led to labile DNA adducts with m/z 299 that increased in a linear fashion over reaction time.^{21a} Upon analysis employing tandem MS, we showed that these adducts fragmented to m/z 152, characteristic of NNK-guanine adducts fragmenting to guanine. Overall, the data were consistent with pyridyloxobutylation of guanine from the bioactivation by cyt P450 2E1.^{21a} Taken as a whole, this demonstrates that the increasing production of HPB in the films is indicative of the increasing population of reactive metabolites in the films leading to increasing amounts of DNA adducts.

Table 1 also summarizes the absorbance peaks from the LC chromatograms based on the calibration curve plot where 0.72 and 0.36 nmol HPB were produced in 10 min, respectively, from cyt P450 1A2 and cyt P450 2E1 films. As Table 1 describes, the LC-MS and ECL array data both show an approximate 50% decrease in enzymatic turnover when going from cyt P450 1A2 to cyt P450 2E1 films, which demonstrates the excellent synergy by both layered film methods to rapidly ascertain and confirm enzyme-substrate specificity.

Discussion

Both ECL array and LC-MS methods employed in this study clearly demonstrate the NNK metabolism differences between the different cyt P450 enzymes. Of the enzymes employed, cyt P450 1A2 exhibited higher specificity toward NNK bioactivation for DNA damage followed by cyt P450 2E1, which was shown to be approximately half as active as cyt P450 1A2 toward NNK metabolism. None of the other enzymes studied here, including cyt P450 1B1, showed preference toward activation of this cancerous compound.

It has been known for some time that the two isoforms (1A2, 2E1) show a capacity toward NNK bioactivation both *in vitro*^{31,32,42} and *in vivo*.^{43,44} The sensor results here showing that cyt P450 1A2 is approximately two times more reactive toward NNK bioactivation for DNA damage than cyt P450 2E1 agree well with previous *in vitro* solution studies. Smith *et al.* employed purified enzymes or heterologously expressed enzymes from different cell lines to study this reaction. Their results have shown that NNK is converted to the keto alcohol (HPB) by cyt P450 1A2 with k_{cat}/K_m values typically 3 to 10 times greater than cyt P450 2E1.^{32,42} Therefore, on a qualitative level, our results correlate well with these previous *in vitro* kinetic studies showing that cyt P450 1A2 exhibits at least twice the

specificity toward NNK bioactivation compared to cyt P450 2E1. Any quantitative deviations (*i.e.* differences in rates, specificity, *etc.*) from previous reports and our results described herein are likely to stem from the different metabolic system used.¹⁵ Here we employed purified enzymes activated via peroxide shunt, which has been shown to produce identical metabolites in varying proportions vs. NADPH activation with P450 reductase.²⁰ Other systems (*i.e.* reconstituted lipid, microsomal, membrane, whole cell, *etc.*) typically generate differences in the Michaelis–Menten parameters.⁴⁵

It is not surprising that cyt P450 1B1 in our sensor did not bioactivate NNK very well as it shows preference for larger aromatic molecules, such as PAHs.^{27b} However, no report to our knowledge has ever suggested that NNK could be metabolized by cyt P450 1B1; therefore, at the very least the use of this enzyme acts as a negative control and confirms previous literature accounts summarizing the enzymes active in the bioactivation of this molecule.^{30b} Overall, depending on the expression system used in addition to the reconstitution parameters, cyt P450 1A2 shows enhanced specificity toward NNK activation compared to cyt P450 2E1.

ECL results can shed some light on the actual *in vivo* toxicity of this molecule. NNK is known to be carcinogenic toward not only the respiratory tract organs but the liver and pancreas as well.^{30,46} Cyt P450 family 2A enzymes are predominantly active in NNK lung metabolism,⁴⁷ but cyt P450 1A2 and cyt P450 2E1 are predominantly located in the liver.^{27b} These two isoforms have been implicated in the complex cancer etiology leading from increasing amounts of NNK metabolites damaging DNA to neoplasia in the liver.^{31,48} Also, these cyt P450 isoforms are inducible through ingestion of other xenobiotics—*i.e.* alcohol consumption promoting increased cyt P450 2E1 expression.⁴⁹ In this vein, hamsters that had been exposed to NNK had a dramatic shift in the locale of new cancerous tissue from lung to pancreas upon additional exposure to alcohol.⁵⁰ While this xenobiotic synergy is not completely understood, one explanation cites the increased quantity of NNK metabolites from the induced cyt P450 2E1 enzymes.⁴³

The chromatographic and ECL methods offered congruent results as both showed that NNK was two times more reactive after interaction with cyt P450 1A2 compared to cyt P450 2E1. The similarity in the LC-MS HPB detection result compared to the ECL turnover values verifies that the ECL signal increase is due to reactive metabolite formation in the films that manifest as detectable DNA adducts. Therefore, this platform offers more than a DNA damage sensor. Rather, it can act as a platform from which toxic metabolite production can be monitored. We previously exhibited this kind of method synergy employing purified enzymes¹⁵ and membrane and/or microsomal enzymes.¹⁷ In the previous report employing purified enzymes, we showed that cyt P450 1B1 was highly specific toward activation of benzo[a]pyrene (B[a]P) followed by cyt P450 1A2 and 2E1. Here, using a different class of molecule, an opposite bioactivation order was obtained, but one that is consistent with previous metabolic studies in the literature. This further demonstrates the utility of the ECL array to be able to rapidly detect enzymatic specificity for a number of potentially hazardous compounds and a range of enzymes. In addition, results also suggest that the ECL/LC-MS method synergy shows great promise for future high-throughput metabolism and toxicity investigations of potential drugs and environmental pollutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1. Cyt P450 catalyzed bioactivation of NNK.³⁰

NNK (1) undergoes α -hydroxylation (2) by various cyt P450 isoforms *in vivo* followed by loss of formaldehyde to yield 4-oxo-4-(3-pyridyl)-1-butane-diazohydroxide (3). 3 is a short lived species that rearranges into highly electrophilic intermediates that are attacked by nucleophilic guanine bases in DNA yielding 7-[4-oxo-4-(3-pyridyl)but-1-yl]Gua (4) or O⁶-[4-oxo-4-(3-pyridyl)but-1-yl]Gua adducts (5) among others. In the absence of DNA, water attacks the electrophilic intermediates yielding the metabolite 4-hydroxyl-1-(3-pyridyl)-1-butanone (HPB, 6).

Fig. 1. Reconstructed array data showing the ECL response from the different DNA/RuPVP/enzyme films exposed to the 1 mM NNK-1 mM H₂O₂ cocktail for the denoted times (s).

Fig. 2.
Relative ECL percentage increase vs. NNK-H₂O₂ reaction time for the enzyme films depicted in Fig. 1.

Fig. 3.

(a) LC chromatograms showing the UV response from the nanoreactor enzyme films upon reaction with NNK and H_2O_2 for 10 min. Control is a 1A2 enzyme film exposed to NNK without the additional H_2O_2 . (b) Mass spectrum of Peak A in (a) demonstrating the presence of material consistent with HPB. (c) Mass spectrum of Peak B in (a) showing the presence of unreacted NNK.

Table 1

Summary of enzyme surface coverage and NNK–enzyme turnover values

Enzyme film	Total surface coverage/pmol cm ⁻²	Initial slope ^a	ECL array turnover value ^b /cm ² (pmol enzyme) ⁻¹ min ⁻¹	LC-UV HPB quant. ^c /nmol
1A2	62(±10)	39.2(±3.70)	0.61(±0.11)	0.72(±0.05)
2E1	54(±8.0)	16.2(±3.10)	0.30(±0.07)	0.36(±0.03)
1B1	52(±6.0)	8.43(±1.45)	0.16(±0.03)	n.d.
CPO	22(±2.0)	3.42(±1.46)	0.16(±0.07)	n.d.

^aFrom Fig. 2 (% ECL increase min⁻¹)^b% ECL increase.^cDetected after 10 min reaction.