Cytochrome P450 mediated oxidation of phenol-type NDGA analogues did not form the expected reactive \textit{para}-quinone methide intermediates suggesting that these analogues might have better safety profile

**MEDICINAL CHEMISTRY | RESEARCH ARTICLE**

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Isaac Asiamah1,† and Ed S. Krol1

Abstract: Reactive metabolites (RMs) have been implicated in many drug-induced toxicities including hepatotoxicity. Nordihydroguaiaretic acid (NDGA) has known pharmacological properties but its use is also associated with toxicities possibly mediated by RMs. In our effort to design and prepare NDGA analogues with better safety profile through rational structural modification, we first evaluated metabolic activation potential of model compounds mimicking catechol- and phenol-type NDGA analogues. We incubated test compounds in rat liver microsomes (RLM) in the presence of glutathione as nucleophilic trapping agent. We also investigated their potential to form para-quinone methides using silver oxide. Glutathione conjugates were detected by electrospray ionization-mass spectrometry (ESI-MS) scanning for neutral loss (NL) 129 or 307 in positive ion mode or precursor ion (PI) scanning for 272 in negative ion mode and further characterized by liquid chromatography–tandem mass spectrometry (LC–MS/MS) or in a single LC-MS run using multiple reactions monitoring (MRM) as a survey scan to trigger acquisition of enhanced

ABOUT THE AUTHORS

Isaac Asiamah Our group is particularly interested in studying the underlying toxicological mechanisms of lignans with the goal of identifying toxicophores to inform rational design of lignan analogues. This forms part of our efforts to discover lignan analogues with better safety profile. In this present study, we first prepared model compounds to mimic catechol- and phenol-type nordihydroguaiaretic (NDGA) analogues and successfully demonstrated by mass spectrometry techniques that phenol-type NDGA analogue are likely to possess better safety profile. We then designed and synthesis a series of catechol- and phenol-type NDGA analogues and evaluated their potential to form reactive intermediates. We are currently evaluating pharmacological properties of all prepared analogues to help establish structure activity relationships. Our goal going forward is to prepare analogues which retain the useful pharmacological properties of NDGA and are not associated with reactive metabolite-mediated toxic liabilities.

PUBLIC INTEREST STATEMENT

The consumption of aqueous extract of creosote bush as Chaparral tea has provided useful health benefits to consumers. However, ingestion of higher doses of the tea has been associated with toxic outcomes. The underlining reasons for the toxic effects of this herbal tea remain unclear. The major component of the tea is known to undergo conversion to reactive metabolites which could potentially cause toxic effects. In this study, we modified the structure of the major component of this tea to assess the effects of various structural modifications on safety profile. Our goal was to discover new compounds with useful health benefits but without toxic liabilities. It was gratifying to observe that one structural modification resulted in a compound which did not form reactive metabolites. This suggests that this new compound may possess better safety profile.
product ion (EPI) data. Our findings led us to subsequently design and synthesize a series of NDGA analogues for evaluating their metabolic activation potential with a goal of eliminating RMs liability. Among others, we found that catechol-type analogues were converted to ortho-quinones by cytochrome P450s. We saw no evidence of RMs by cytochrome P450s for phenol-type analogues. This suggest that phenol-type NDGA analogues might not be associated with reactive metabolites-mediated toxicities. Although a more extensive pharmacological evaluation is underway, our preliminary results revealed that pharmacological properties were not compromised.

**Subjects:** Medicinal chemistry; Toxicology; Pharmacology

**Keywords:** reactive metabolites; glutathione conjugates; triple quadrupole-linear ion trap mass spectrometry; neutral loss; quinones; rational drug design

### 1. Introduction

The aqueous extract of Creosote bush known as Chaparral tea was listed by the FDA as an ethnobotanical used to treat tuberculosis, arthritis and cancer (Tyler, 1994). The lignan nordihydroguaiaretic acid (NDGA) is the main secondary metabolite of this shrub and is reported to have numerous pharmacological properties (Lambert, Dorr, & Timmermann, 2004). NDGA continues to attract research attention due to its promising applications in the treatment of cardiovascular diseases, neurological disorders and cancers. NDGA has been reported to inhibit numerous viruses including human immunodeficiency virus (HIV-1), herpes simplex virus (HSV), human papilloma virus (HPV) and influenza virus (Craig, Callahan, Huang, & Delucia, 2000; Lambert et al., 2004). NDGA possesses radical scavenging (Floriano-Sanchez et al., 2006; Galano, Macias-Ruvalcaba, Medina Campos, & Pedraza-Chaverri, 2010) and antioxidant properties (Sheikh, Philen, & Love, 1997) as well as anti-inflammatory (Bhattacherjee et al., 1988) and anti-proliferative properties.

We previously reported NDGA autoxidation at physiologically relevant conditions to a dibenzocyclooctadiene lignan (Billinsky & Krol, 2008) with potential pharmacological implications. In spite of its broad pharmacological activities, NDGA use is associated with toxicity especially when ingested at higher doses (Batchelor, Heathcote, & Wanless, 1995; Sheikh et al., 1997). Reports of severe hepatic (Batchelor et al., 1995; Gordon, Rosenthal, Hart, Sirota, & Baker, 1995; Sheikh et al., 1997; Stickel, Egerer, & Seitz, 2000) and renal injuries (Goodman, Grice, Becking, & Salem, 1970; Grice, Becking, & Goodman, 1968; Smith, Feddersen, Gardner, & Davis, 1994) are likely mediated by bioactivation to reactive quinones (Scheme 1). We (Billinsky, Marcoux, & Krol, 2007) and others (Goodman et al., 1970; Grice et al., 1968) have reported on oxidation of NDGA to ortho-quinones but no evidence of para-quinone methides formation was observed although structurally similar secoisolariciresinol (SECO) converts to lariciresinol (Billinsky et al., 2007; Cavalieri et al., 2002) possibly via a para-quinone methide intermediate (Scheme 2).

Early detection and characterization of reactive intermediates allow structural optimization of new chemical entities to minimize or eliminate the potential for bioactivation and reduce attrition at later stages of development. In our efforts to understand the structural features which influence reactive metabolites formation for dibenzylbutane-like lignans, we synthesized a series of catechol-type (4, 7, 8) and phenol-type (5, 6) analogues of NDGA (Asiamah, Hodgson, Maloney, Allen, & Krol, 2015) (Figure 1) for the purpose of studying their oxidative metabolism to quinones. We hypothesized that formation and type of quinoid species is dependent on substitution and/or substitution pattern of the aromatic rings. We expected the catechol-type analogues to form both ortho-quinones and para-quinone methides whilst the phenol-type analogues were predicted to form only para-quinone methides under oxidative conditions. The compounds were subjected to chemical and enzymatic oxidations and the resulting reactive intermediates stably trapped as glutathione (GSH) adducts. GSH typically forms adducts of ortho-quinones through addition to the
One of the challenges to rapid screening for GSH adducts of ortho-quinones vs para-quinone methides is isolation and purification of sufficient quantities of the adduct for analysis by nuclear magnetic resonance (NMR) spectrometry. Mass spectrometry (MS) continues to be an attractive tool in the drug discovery and development process due to its high sensitivity, selectivity and high throughputs. In an effort to assess quinone formation in lignans with limited starting material, we investigated the utility of an electrospray tandem mass spectrometry (ESI-MS/MS) technique to distinguish between aromatic and benzylic GSH adducts (Scheme 3) (Xie, Zhong, & Chen, 2013). To achieve this objective, we investigated the ESI-MS/MS properties of model compounds (1, 2 and 3; Figure 1) known to form ortho-quinones or para-quinone methides by searching for diagnostic product ions for the corresponding aromatic and benzylic GSH adducts. The model compounds were oxidized both chemically and enzymatically and the resulting quinones were stably trapped as GSH conjugates for MS experiments. ESI-MS analyses revealed diagnostic ions for benzylic GSH adducts (NL 307) and GSH ring adducts (NL 129, precursor ion (PI) m/z 272). We then applied this methodology to analyse the GSH adducts of our NDGA analogues to distinguish between ring and benzylic adducts. These findings allow for rapid profiling of new chemical entities capable of forming ortho-quinones and/or para-quinone methides and help to reduce cost and labour-intensive isolations for NMR analysis. The results of this study directed our
efforts at designing phenol-type NDGA analogues which formed no reactive metabolites with cytochrome P450s and suggestive of better safety profiles.

2. Materials and methods

General information: meso-Nordihydroguaiaretic acid (NDGA, 97%) from Larrea tridentata, reduced glutathione (GSH), Tyrosinase from mushroom (EC1.14.18.1), dimethylsulfoxide (DMSO), NADP(H), AgNO₃ and K₂HPO₄ were purchased from Sigma-Aldrich Co. (Madison, WI). Citric acid and HCl were purchased from Fisher Scientific (Ottawa, ON). Formic acid was purchased from BDH. All solvents were HPLC grade. Water was purified via a Millipore (Mississauga, ON) Milli-Q system with a Quantum EX Cartridge. Synthetic details of NDGA analogues studied in this work have been published elsewhere (Asiamah et al., 2015).
Instrumentation: HPLC system consisted of Waters Alliance 2695 connected to Waters 2996 photodiode array detector. The HPLC system was controlled by, and data interpreted with the Waters Empower software. An Allsphere ODS-2 microbore column (3 µm, 150 × 2.1 mm) operating at a flow rate of 0.2 ml/min was used to run a gradient elution. Solvent A: 0.1% formic acid/H₂O, solvent B: 0.1% formic acid/CH₃CN. An initial isocratic phase of 90% A for 2 min was decreased to 60% A over 8 min and further to 10% A over 12 min, then held isocratic at 10% A for 10 min and finally increased to 90% A over 1 min and held isocratic at 90% A for 6 min to equilibrate the column. MS experiments were conducted using AB SCIEX 4000 QTRAP (AB SCIEX instruments) quadrupole linear ion trap mass spectrometer coupled to an Agilent 1100 system consisting of an Agilent 1100 G1311A pump and an Agilent 1100 G1329A autosampler (Agilent Technologies, Mississauga, ON). Data acquisition and analysis were performed using Analyst 1.5.1 software from AB SCIEX.

2.1. Model compounds
Hydroxychavicol 1, 2-methoxy-4-propylphenol 2 and 4-allyl catechol 3 (Figure 1) were prepared from eugenol by following literature procedures (Bolton et al., 1994, 1996) and used as model compounds. Compound 2 mimics the phenol-type NDGA analogues while compounds 1 and 3 mimic the catechol-type NDGA analogues under oxidative conditions.

2.2. Enzymatic oxidation studies
Each test compound (1 mM), glutathione (5.0 mM) and mushroom tyrosinase (47.25 units) in 500 µl Na₂HPO₄ buffer (50 mM, pH 6.0) was vortexed for 60 min at room temperature (25°C). The reaction was stopped by addition of perchloric acid (20 µl). After centrifugation (14,000 rpm for 10 min), aliquots of the supernatant were analyzed directly by HPLC and further by ESI-MS. Control experiments were performed in the absence of GSH, in the absence of mushroom tyrosinase, and with the addition of GSH after 60 min vortex. Each reaction was performed in triplicate.

2.3. Chemical oxidation studies
Freshly prepared silver oxide (300 mg) was added to the test sample (2.5 mM) in dried acetonitrile pre-equilibrated to 60°C. Aliquots (1 ml) were taken at 30 s or 30 min and centrifuged at 14,000 rpm for 10 min. The resulting supernatant was added to a potassium phosphate buffer (pH 7.4) containing GSH to a final concentration ratio of 1:5 substrate-GSH. Control experiments contained no GSH and were all performed in duplicates. The samples were analyzed directly by HPLC. For MS analysis, samples were loaded on C-18 extraction cartridges (Bond Elut, Varian) and eluted with methanol.

2.4. Cytochrome P450 oxidation studies
Test compounds in DMSO (0.5 mM) were incubated in Na₂HPO₄ buffer (50 mM, pH 7.4) containing GSH (5 mM), rat liver microsomes (0.5 mg/ml) and MgCl₂ at 37°C for 60 min. The final reaction volume (500 µl) contained 0.5% DMSO solvent. The reaction was initiated with NADPH after 5 min pre-incubation and terminated by chilling in ice bath followed by addition of ice-cold acetonitrile (200 µl). After centrifugation at 14,000 rpm for 10 min, the supernatant was analysed by HPLC and LC-MS. Samples for LC-MS were loaded on C-18 extraction cartridges (Bond Elut, Varian) and eluted with methanol. Control experiments contained no NADPH. All experiments were run in duplicates.

2.5. Mass spectrometric analysis
Each sample was diluted 100 times in methanol prior to MS analysis. Samples were infused into the QqQLT-MS at the flow rate of 10 µL/min using a Harvard Syringe Pump or by HPLC. Enhanced resolution (ER) scans, neutral loss (NL) scans, precursor ion (PI) scans, MS/MS scans and multiple reaction monitoring-enhanced product ion (MRM-EPI) scans were performed to provide information for structural characterization and fragmentation pathways. Parameters for MS/MS analysis were optimized to ensure the formation of the product ions while maintaining the presence of the precursor ion.
3. Results

3.1. Detection of glutathione conjugates by neutral loss (NL) and precursor ion (PI) scanning techniques

The goal of this study was to understand the nature of quinones formed by catechol and phenol NDGA analogues to help design analogues with better safety profile through rational structural modification. We carried out two oxidations to prepare quinones from our catechol and phenol analogues, using mushroom tyrosinase and silver oxide as our oxidizing systems. Phenols are known to oxidize to para-quinone methides by silver oxide whereas catechols form ortho-quinones when oxidized with either mushroom tyrosinase or silver oxide (Bolton et al., 1994, 1996). Some ortho-quinones can undergo isomerization to para-quinone methides (Bolton et al., 1994), although we did not observe this for NDGA (Billinsky et al., 2007). We then trapped resulting quinones with a fivefold excess of GSH.

We first carried out ESI-MS experiments on our model compounds 1, 2 and 3 for which GSH adducts have previously been characterized (Bolton et al., 1994, 1996). To screen for the formation of GSH ring adducts we searched for a neutral loss of 129 Da (loss of a pyroglutamate moiety) in positive ionization mode (Scheme 4(a)) and we also conducted a precursor ion (PI) scan in negative ionization mode at m/z 272 Da (loss of GSH minus sulfur) (Scheme 4(b)) (Dieckhaus, Fernández-Metzler, King, Krolikowski, & Baillie, 2005). To screen for the formation of benzylic GSH adducts, we searched for a neutral loss of 307 Da (loss of an intact GSH moiety) in positive ionization mode (Scheme 4(c)). ESI-MS experiments using neutral loss scan in positive ionization mode for ortho-quinones forming catechols 1 and 3 showed the anticipated neutral loss of 129 Da, yielding parent ions at m/z 458.2 and 456.1 Da, respectively (Figure 2). Compounds 1 and 3 also yielded parent ions at m/z 456.2 and 454.2 Da, respectively, by PI scan for m/z 272 in negative ion mode (Table 1). No parent ion was observed for compound 1 by NL scan for 307 in positive ion mode. Conversely, NL scanning for para-quinone methide forming phenol 2 in positive ion mode produced a product ion at m/z 470.1 by NL 307. We observed no product ion by NL 129 scanning in positive ion mode experiments. We also found no evidence of glutathione adduct by PI scan for m/z 272 in negative ion mode for compound 2, suggesting this approach could distinguish between ring and benzylic adducts. The GSH adduct of catechol 3 was however detectable by both NL 129 and 307 scanning experiments, although the NL 129 signal was stronger. This was not unexpected as the allylic substituent on compound 3 results in rapid isomerization from the ortho-quinone to the para-quinone methide (Bolton et al., 1994).

Neutral loss analysis on our NDGA oxidation experiments with either mushroom tyrosinase or silver oxide yielded parent ion at m/z 608.3 by NL 129 but not NL 307 in positive ionization mode (Figure 2 panel B). Also, a PI scan for m/z 272 in negative ion mode gave a consistent parent ion at m/z 606.3 Da (Table 1). Further, ESI-MS/MS experiments afforded a product ion at m/z 479 (Figure 3 panel A) consistent with NL of 129 but we observed no fragment ions corresponding to NL of 307.

Scheme 4. MS detection of glutathione conjugates by (a) NL scanning for 129 Da in positive ion mode; (b) PI scanning for m/z 272 Da in negative ion mode and (c) NL scanning for 307 Da in positive ion mode.
Figure 2. ESI-MS analysis using NL 129 or 307 in positive ionization scanning mode for detection of glutathione conjugates. Adducts generated by mushroom tyrosinase, silver oxide as well as rat liver microsomes oxidations for model compound 1 (panel A), NDGA (panel B) and catechol-type NDGA analogues 4 (panel C), 7 (panel D) and 8 (panel E) were detectable by NL 129 but not NL 307 experiments. Adducts following repeated trapping experiments with model compound 2 (panel F) and phenol-type analogue 5 (panel G) were blind to NL 129 but easily detectable by NL 307 MS experiment. Adducts of compound 3 were detected by both NL 129 (panel H) and NL 307 (panel I) experiments. Phenol-type analogue 6 gave no evidence of adduct formation by NL 129 or NL 307 Da MS experiments.

Table 1. Detection of GSH adducts using different ESI-MS scanning techniques

| Compound–GSH conjugate (M, Da) | MS detection technique |
|--------------------------------|------------------------|
|                                | ESI-MS (+) NL 129 | ESI-MS (+) NL 307 | ESI-MS (-) PI 272 |
| 1–GSH (457)                    | 458.1                | ND                | 456.2 |
| 2–GSH (471)                    | ND                   | 472.2             | ND    |
| 3–GSH (455)                    | 456.1                | 456.1             | 454.2 |
| NDGA–GSH (607)                 | 608.3                | ND                | 606.3 |
| 4–GSH (575)                    | 576.1                | ND                | 574.2 |
| 5–GSH (589)                    | ND                   | 590.2             | ND    |
| 6–GSH (635)                    | ND                   | ND                | ND    |
| 7–GSH (635)                    | 636.2                | ND                | 634.2 |
| 8–GSH (579)                    | 580.4                | ND                | 578.2 |

ND = not detected

(i.e. m/z 301), confirming that only the anticipated ortho-quinone-derived GSH ring adducts observed previously (Billinsky et al., 2007) were present. Similar to NDGA, we observed that NDGA analogue 8 (no methyl groups on the linker) produced only GSH ring adducts corresponding to ortho-quinone formation (parent ion at m/z 580.4 by NL 129 scan in positive ion mode; parent ion at m/z 578.2 by PI m/z 272 scan in negative ion mode; ESI-MS/MS product ion at m/z 505 Da). We then applied this approach to our remaining catechol and phenol NDGA analogues.
The presence of m/z 308.1 Da for excess GSH was confirmed by NL 129 analysis on standard GSH. We first carried out mushroom tyrosinase oxidations for which NL 129 scanning in positive ion mode resulted in parent ions at m/z 576.1 and 636.3 Da, consistent with the formation of mono-GSH ring conjugates of the catechols 4 and 7 respectively. NL 129 experiments gave no evidence for GSH ring conjugates for compounds 5 or 6 following mushroom tyrosinase oxidation. After chemical oxidation with silver oxide, we observed that four of the NDGA analogues were converted to their respective GSH adducts in the trapping experiments. However, these adducts behaved differently in the NL detection experiments. The adduct generated from 5 produced no parent ion by NL 129 scan in positive ion mode or PI m/z 272 scan in negative ion mode but was easily detected by NL 307 scan in positive ion mode (Figure 2 panel G, Table 1). Adducts formed from 4, 7 and 8 behaved similarly to their respective adducts from mushroom tyrosinase oxidation when subjected to NL 129 or NL 307 experiments.

3.2. Enhanced resolution ESI-MS analysis
To confirm the presence of GSH conjugates we first used ESI-MS in enhanced resolution (ER) positive ionization scanning mode. This gave parent ions (MH⁺) and isotopic peaks separated by one mass unit indicating singly charged adducts (Tables 2 and 3). Doubly charged adducts will be expected to have one-half mass unit difference between isotopic peaks. For the mushroom tyrosinase oxidations, we observed ions consistent with the addition of one molecule of GSH to NDGA, 1, 3, 4, 7 and 8 (Table 2). We detected no GSH adducts for the phenol analogues treated with mushroom tyrosinase. Following silver oxide treatment, GSH adducts were observed for NDGA, 1–5, 7 and 8. Curiously, we did not detect any GSH adducts for the di-phenol 6 (Table 3).

3.3. Tandem ESI-MS/MS analyses
To obtain further information on the structure of the GSH adducts, we subjected the samples to tandem ESI-MS/MS analyses. The product ions generated by MS/MS analyses (Figure 3, Tables 2–4) were consistent with fragmentation patterns reported for glutathione adducts (Dieckhaus et al., 2005; Xie et al., 2013) and mirrored those obtained for standard GSH. The positive ion mode
Table 2. Summary of tandem MS/MS data for GSH conjugates following mushroom tyrosinase-catalysed oxidation

| Compound (M) | MH\(^+\) and (major fragments) of GSH adduct\(^b\) | Adduct composition | Isotopic peaks\(^c\) |
|--------------|--------------------------------------------------|-------------------|---------------------|
| NDGA (302)   | \(^608\) (533, 479, 462, 387, 376, 359 333, 130) | P + GSH—2H        | 608.1, 609.1, 610.1 |
| 1 (152)      | \(^458\) (383, 329, 312, 237, 266, 183, 130)    | P + GSH—2H        | 458.1, 459.1, 460.1 |
| 2 (166)      | -                                                | -                 | -                   |
| 3 (150)      | \(^456\) (381, 327, 310, 235, 264, 181, 130)    | M + GSH—2H        | 456.1, 457.1, 458.1 |
| 4 (270)      | \(^656\) (501, 447, 430, 355, 344, 327, 301, 130) | P + GSH—2H        | 576.1, 577.1, 578.1 |
| 5 (284)      | -                                                | -                 | -                   |
| 6 (330)      | -                                                | -                 | -                   |
| 7 (330)      | \(^636\) (561, 507, 490, 415, 404, 387, 361, 130) | P + GSH—2H        | 636.2, 637.2, 638.2 |
| 8 (274)      | \(^580\) (505, 451, 434, 359, 348, 331, 305, 130) | P + GSH—2H        | 580.1, 581.1, 582.1 |

\(^a\)The GSH adducts detected were the peaks detected by NL scan for 129 Da.

\(^b\)Product ions listed were greater than 5% relative intensity. The boldface type denotes the product ions from the loss of 129 Da mass units from the parent ion.

\(^c\)Isotopic peaks were obtained by ER in positive ion scanning mode.

Table 3. Summary of tandem MS/MS data for GSH conjugates following silver oxide oxidation experiment

| Compound (M) | MH\(^+\) and (major fragments) of GSH adduct\(^d\) | Adduct composition | Isotopic peaks\(^g\) |
|--------------|--------------------------------------------------|-------------------|---------------------|
| NDGA (302)   | \(^608\) (479, 462, 376, 359, 333, 145, 130)       | P + GSH—2H        | 608.1, 609.1, 610.1 |
| 1 (152)      | \(^458\) (329, 312, 226, 209, 183, 145, 130)       | P + GSH—2H        | 458.1, 459.1, 460.1 |
| 2 (166)      | \(^472\) (308, 233, 179, 165)                     | P + GSH—2H        | 472.2, 473.2, 474.2 |
| 3 (150)      | \(^456\) (327, 310, 224, 207, 181, 149, 130)       | M + GSH—2H        | 456.2, 457.2, 458.2 |
| 4 (270)      | \(^576\) (501, 447, 430, 355, 344, 327, 301, 130) | P + GSH—2H        | 576.1, 577.1, 578.1 |
| 5 (284)      | \(^490\) (308, 283, 159, 179, 137)                | -                 | -                   |
| 6 (330)      | -                                                | -                 | -                   |
| 7 (330)      | \(^636\) (561, 507, 490, 415, 404, 387, 343, 327, 145) | P + GSH—2H        | 636.2, 637.2, 638.2 |
| 8 (274)      | \(^580\) (451, 442, 331, 305, 295, 287, 199, 163, 130) | P + GSH—2H        | 580.2, 581.2, 582.2 |

\(^a\)The GSH adducts detected were the peaks detected by NL scan for 307 Da in positive ion mode only.

\(^b\)The GSH adducts were detected by either NL scan for 129 Da in positive ion mode or PI scan for m/z 272 Da in negative ion mode.

\(^c\)The GSH adduct was detected by both NL 129 and 307 Da scan in positive ion mode as well as PI 272 scan in negative ion mode.

\(^d\)The product ions were obtained in positive ion mode and the major ions listed were greater than 5% relative intensity.

\(^e\)The product ions from the loss of 307 mass unit from the parent ions.

\(^f\)The product ions from the loss of 129 Da mass unit from the parent ion.

\(^g\)Isotopic peaks were obtained by ER in positive ion scanning mode.
fragmentation pathways (Figure 4) were consistent for all GSH conjugates examined including NDGA (Figure 3A). The mass loss patterns and the resulting product ions suggest that fragmentation mainly occurred in the GSH moiety consistent with previous reports (Dieckhaus et al., 2005; Xie et al., 2013). The common neutral losses of glycine (75 Da), pyroglutamic acid (129 Da) and glutamic acid (146 Da) observed for standard GSH (data not shown) were also seen for GSH conjugates of NDGA, 4, 7 and the NDGA catechol analogues (4, 7 and 8). In addition, other typical losses from the parent ion MH+ including 232, 249 and 275 Da mass units were consistently observed for all GSH conjugates analyzed (Tables 2–4).

3.4. MRM-EPI MS analyses

The use of multiple reactions monitoring (MRM) as a survey scan to trigger acquisition of enhanced product ion (EPI) provides superior sensitivity and selectivity for GSH adducts (Xie et al., 2013). We incubated NDGA and its prepared analogues with rat liver microsomes (RLM) and the resulting reactive metabolites were trapped as glutathione conjugates. MRM-EPI experiments afforded product ions consistent with aromatic conjugates and in agreement with standards we generated in the mushroom tyrosinase experiments for NDGA, 4, 7 and 8. As shown in Figure 5, the presence of d/k-type ions resulting from cleavage of the cysteiny1 C-S bond as well as product ions mainly derived from cleavage of bonds within the glutathionyl moiety including a-, b-, c-, d-, e-, f-, g- and h-type ions are all consistent with aromatic conjugate. We found no evidence of glutathione conjugates of the phenol NDGA analogues by MRM-EPI analysis.

4. Discussion

The mechanisms of NDGA toxicity remain unclear although quinone formation has been implicated in hepatotoxicity (Walgren, Mitchell, & Thompson, 2005). NDGA can undergo oxidation to either an ortho-quinone or a para-quinone methide, either of which could contribute to hepatotoxicity. Such transformations may be cytochrome P450-mediated or catalysed by other oxidases. We (Billinsky et al., 2007) and others (Goodman et al., 1970; Grice et al., 1968) have reported evidence of NDGA oxidation to an ortho-quinone although no evidence of para-quinone methide formation has been reported. Furthermore, observation that the lignan secosolariciresinol appears to undergo an intramolecular cyclization to lariciresinol via a para-quinone methide led us to investigate whether this was a viable reaction for NDGA-like lignans (Niemeyer, Honig, Kulling, & Metzler, 2003). Our efforts to understand the potential toxicological mechanisms of NDGA inspired us to investigate the nature of quinoid species formed by NDGA and its analogues (Figure 1) under oxidative conditions. To further explore why NDGA does not seem to form para-quinone methide, we
Figure 4. Typical fragmentation pathways for GSH conjugates following CID for dibenzylbutane-type lignans. P represents the parent ion (MH⁺).

| Fragment type | Positive m/z |
|---------------|--------------|
| a             | P-75         |
| b             | 145          |
| c             | P-146        |
| d             | P-275        |
| e             | P-129        |
| f             | 130          |
| g             | P-232        |
| h             | P-249        |
| i             | 308          |
| j             | P-307        |
| k             | 274          |

Figure 5. MRM-EPI analysis of GSH conjugates formed in the rat liver microsomes (RLM) incubation for 7. XIC (panel A); MRM (panel B) and EPI (panel C).
designed and prepared NDGA analogues (Asiamah et al., 2015) and subjected them to P450 mediated oxidation studies in the presence of glutathione. Standards for comparison were generated by glutathione trapping experiments with chemical (Bolton et al., 1994, 1996) and enzyme (Bolton et al., 1994; Iverson, Hu, Vukomanovic, & Bolton, 1995; Krol & Bolton, 1997) oxidation systems. We identified the nature of quinoid species indirectly from the type of glutathione adduct formed in our nucleophilic trapping experiments. We expected GSH to bind covalently at an aromatic ring carbon of an ortho-quinone to give aromatic GSH adduct. Conversely, GSH binding to para-quinone methide would occur exocyclic to the ring carbon to yield a benzylic adduct (Scheme 3) (Xie et al., 2013).

LC-MS/MS techniques have been routinely utilized in in vitro evaluations of new chemotypes for reactive metabolites via GSH trapping experiments (Baillie, Pearson, Rashed, & Howald, 1989). We detected glutathione adducts by NL 129 or 307 in positive ionization mode and precursor ion (PI) scanning for m/z 272 Da in negative ionization mode (Dieckhaus et al., 2005) (Scheme 4). Conjugates for model compounds 1 and 3, NDGA and NDGA catechol analogues were detected by NL 129 or PI m/z 272 but not NL 307 scanning. Glutathione conjugates of compound 2 and NDGA phenol analogues on the other hand were blind to NL 129 or PI m/z 272 but were detected by NL 307 (Table 1, Figure 2). Neutral loss and precursor ion scanning techniques have been described in the literature for detecting different classes of GSH adduct (Dieckhaus et al., 2005; Zheng et al., 2007) although general applicability is limited. We optimized the substrate to GSH ratio (1:5) to favour addition of one glutathione molecule (Billinsky et al., 2007) as observed by enhanced resolution (ER) MS experiments (Tables 2–4). Isotopic peaks obtained by ESI-ER MS were separated by one mass unit indicating singly charged adducts. Doubly charged adducts will be expected to have one-half mass unit difference between isotopic peaks.

Tandem MS/MS experiments provided further information to help to determine the structures for all suspected glutathione adducts (Figure 3, Tables 2–4). It was gratifying to observe the presence of product ion at m/z 130 Da for adducts of 1, 3, NDGA and its catechol analogues while adducts of 2 and phenol-type analogue S yielded m/z 308 Da following collision-induced dissociation (CID) in MS/MS experiments. This suggests loss of the pyroglutamate moiety by conjugates of 1, 3, NDGA or its catechol-type analogues whilst phenols 2 or 5 lost a glutathione moiety consistent with NL 129 and NL 307 experiments respectively. The typical fragmentation patterns observed for GSH conjugates of NDGA and its analogues are summarized in Figure 4. The product ions are indicated by α- to k- in accordance with the literature (Baillie et al., 1989; Haroldsen, Reilly, Hughes, Gaskell, & Porter, 1988; Xie et al., 2013). Fragmentation-based methods have been used to differentiate glutathione conjugates belonging to different structural classes (Xie et al., 2013). In particular, the cleavage of the cysteiny1 C-S bond leading to formation of d/k-type ions is diagnostic for aromatic conjugates whereas benzylic conjugates primarily yield i/j-type ions upon cleavage of the C-S bond between the test compound and GSH. The strong π→backbonding interaction between the lone-pair electron of the glutathionyl sulfur atom and the aromatic ring has been suggested to increase the resistance of the C-S bond between the test compound and GSH to CID. It is worth noting that the d-type ion formed by the cleavage of the cysteiny1 C-S bond and characteristic of ring adducts (Xie et al., 2013) was observed for NDGA and all its catechol-type analogues. Additionally, e-type product ion resulting from loss of pyroglutamic acid moiety is indicative of aromatic conjugates. Therefore, P450-mediated bioactivation of NDGA and its catechol-type analogues form aromatic conjugates derived from ortho-quinones. Typical losses in product ion scan including 75, 129, 146, 249 and 275 mass units were observed for all aromatic conjugates. The only evidence of benzylic conjugates which primarily yield i/j-type ions upon cleavage of the C-S bond between the test compound and GSH (Xie et al., 2013) was seen for 2 and phenolic-type analogue 5 following Ag2O catalyzed oxidation.

Neutral loss of 129 Da is used widely for detecting GSH adducts, especially when protonated molecules of GSH conjugates cannot be predicted, but it suffers low LC/MS sensitivity and limited selectivity resulting from the interference of endogenous compounds and background noise. Also,
NL fragmentation patterns are compound-dependent making the effectiveness of NL experiments vary among different classes of GSH adducts. Selectivity of NL scanning for GSH adducts can be improved in a number of way including a) the use of a mixture of GSH and stable-isotope labelled GSH (1:1 ratio) as trapping agent (Yan & Caldwell, 2004; Yan, Maher, Torres, Caldwell, & Huebert, 2005). b) high-resolution LC/MS (Castro-Perez, Plumb, Liang, & Yang, 2005). c) a multiple reaction monitoring (MRM) method to monitor typical transitions of protonated (MH⁺) potential reactive metabolites trapped as GSH adducts (Soglia et al., 2004) and d) precursor ion (PI) scanning in negative electrospray ionization mode (Dieckhaus et al., 2005). An MRM-based approach provides superior sensitivity and selectivity for GSH adducts, especially where isotope labelled GSH is not available, but it only detects the GSH adducts pre-set on an MRM transition protocol (Zheng et al., 2007). To further confirm RMs formed from P450-mediated oxidation, MRM was used as a survey scan to trigger acquisition of EPI. As shown in Figure 5 for compound 7, the findings were consistent with NL 129 scanning and tandem MS/MS studies. Also, the NDGA-derived dibenzyoclooctadiene lignan (cNDGA) underwent P450-mediated oxidation to reactive quinones as determined by ESI-MS/MS experiment on the glutathione-trapped adduct (Table 4) suggesting that it potentially contributes to NDGA toxicity. This observation agrees with findings by Galano et al. (2010) who reported that NDGA forms a cyclic compound which can be further oxidized, although at a higher potential than NDGA.

One unexpected finding of our study was the inability to form GSH adducts from Ag₂O oxidation of the di-phenol compound 6 (dihydroguaiaretic acid) which is a direct comparator to the lignan secoisolariciresinol, which has been suggested to form lariociresinol through a para-quinone methide intermediate (Niemeyer et al., 2003). Oxidation of 4,4ʹ-butane-1,4-diydiphenol, an analogue of 6, with potassium ferricyanide is reported to form dispirotetraenedione through intramolecular cyclization mediated by di-radical species (Scheme 5) (Afzal, Jordaan, & Kirby, 1969). If oxidation of compound 6 occurs preferentially at both phenols, then intramolecular cyclization to a dispirotetraenedione may occur more rapidly than oxidation at the benzylic carbon to yield a para-quinone methide. We and others have previously observed that secoisolariciresinol unexpectedly undergoes preferential oxidation on one ring rather than at both phenols (Eklund et al., 2005; Hosseinian, Muir, Westcott, & Krol, 2007). This is in contrast with the NDGA lignans and suggests that para-quinone methide formation and GSH adduct formation for the NDGA lignans is precluded in a symmetrical phenol.

5. Conclusions
Oxidative metabolism and bioactivation studies on NDGA and its analogues confirmed that reactive metabolite formation is dependent on the presence of catechol or phenol moieties. Cytochrome P450-mediated oxidation of NDGA and its catechol-type analogues yielded electrophilic intermediates which reacted with GSH. LC-ESI-MS analysis in positive ionization mode gave parent ions consistent with addition of one GSH molecule to NDGA or its catechol-type analogues. A further tandem MS/MS studies gave product ions consistent with typical fragmentation patterns reported for GSH conjugates (Dieckhaus et al., 2005; Xie et al., 2013; Zheng et al., 2007). The fragmentation patterns were used to identify the GSH conjugates as ring adducts derived from ortho-quinones although the position of attachment on the aromatic ring could not be determined. We also found that NL 129 or 307 scanning in positive ionization mode has potential diagnostic utility as a rapid screening method for distinguishing between aromatic and benzylic GSH.

Scheme 5. Proposed intramolecular cyclization of compound 6 to a dispirotetraenedione (Afzal et al., 1969).
conjugates. We found no evidence of para-quinone methide formation suggesting that an ortho-quinone is the major reactive toxicophore responsible for reactive metabolite mediated toxicities associated with NDGA use. In addition, we found that the NDGA-derived dibenzocyclooctadiene lignan (cNDGA) undergoes P450-mediated oxidation to a reactive metabolite which might have toxicological implications. There was no evidence of P450-mediated oxidation to reactive metabolites for the phenol-type NDGA analogues suggesting that phenol-type analogues might have a better safety profile, although further work is needed. Finally, dihydroguaiaretic acid does not form the expected para-quinone methide, suggesting that the methylene hydroxy groups present in the linker of secoisolariciresinol have a major influence on the reactivity of lignans. It appears that for symmetrical lignans which do not possess methylene hydroxyl groups the phenol/catechol rings have identical oxidation properties, whereas for lignans possessing methylene hydroxyl groups the phenol’s one ring can oxidize preferentially to the other.

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Competing Interest
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Author details
Isaac Asiamah1
E-mail: isaac.asiamah@usask.ca
ORCID ID: http://orcid.org/0000-0003-3451-4379
Ed S. Krol2
E-mail: ed.krol@usask.ca

1 Drug Discovery and Development Research Group, College of Pharmacy and Nutrition, University of Saskatchewan, 107 Wiggins Rd, Saskatoon, SK S7N 5E5, Canada.
2 Current address: Department of Chemistry, School of Physical Sciences, College of Agriculture and Natural Sciences, University of Cape Coast, Cape Coast, Ghana
E-mail: aiasiamah1@ucc.edu.gh.

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