Quinoid derivatives of the nevirapine metabolites 2-hydroxy- and 3-hydroxy-nevirapine: activation pathway to amino acid adducts†

Shrika G. Harjivan,[a] Pedro F. Pinheiro,[a] Inês L. Martins,[a] Ana L. Godinho,[a] Riccardo Wanke,[a] Pedro P. Santos,[a] Sofia A. Pereira,[b] Frederick A. Beland,[c] M. Matilde Marques*[a] and Alexandra M. M. Antunes*[a]

Nevirapine (NVP) is the non-nucleoside HIV-1 reverse transcriptase inhibitor most commonly used in developing countries, both as a component of combined antiretroviral therapy and to prevent mother-to-child transmission of the virus; however, severe hepatotoxicity and serious adverse cutaneous effects raise concerns about its safety. NVP metabolism yields several phenolic derivatives conceivably capable of undergoing further metabolic oxidation to electrophilic quinoid derivatives prone to react with bionucleophiles and initiate toxic responses. We investigated the ability of two phenolic NVP metabolites, 2-hydroxy-NVP and 3-hydroxy-NVP, to undergo oxidation and subsequent reaction with bionucleophiles. Both metabolites yielded the same ring-contraction product upon oxidation with Frémy's salt in aqueous medium. This is consistent with the formation of a 2,3-NVP-quinone intermediate, which upon stabilization by reduction was fully characterized by mass spectrometry and nuclear magnetic resonance spectroscopy. Additionally, we established that the oxidative activation of 2-hydroxy-NVP involved the transient formation of both the quinone and a quinone-imine, whereas 3-hydroxy-NVP was selectively converted into 2,3-NVP-quinone. The oxidations of 2-hydroxy-NVP and 3-hydroxy-NVP in the presence of the model amino acids ethyl valinate (to mimic the highly reactive N-terminal valine of hemoglobin) and N-acetylcysteine were also investigated. Ethyl valinate reacted with both 2,3-NVP-quinone and NVP-quinone-imine, yielding covalent adducts. By contrast, neither 2,3-NVP-quinone nor NVP-derived quinone-imine reacted with N-acetylcysteine. The product profile observed upon Frémy's salt oxidation of 2-hydroxy-NVP in the presence of ethyl valinate was replicated with myeloperoxidase-mediated oxidation. Additionally, tyrosinase-mediated oxidations selectively yielded 2,3-NVP-quinone-derived products, while quinone-imine-derived products were obtained upon lactoperoxidase catalysis. These observations suggest that the metabolic conversion of phenolic NVP metabolites into quinoid electrophiles is biologically plausible. Moreover, the lack of reaction with sulfhydryl groups might hamper the *in vivo* detoxification of NVP-derived quinone and quinone-imine metabolites *via* glutathione conjugation. As a result, these metabolites could be available for reaction with nitrogen-based bionucleophiles (*e.g.*, lysine residues of proteins) ultimately eliciting toxic events.

[a]*Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal. E-mail: matilde.marques@tecnico.ulisboa.pt, alexandra.antunes@tecnico.ulisboa.pt; Fax: +351 218 464 455; Tel: +351 218 419 200, +351 218 417 627*
[b]*Centro de Estudos de Doenças Crónicas (CEDOC), NOVA Medical School, Universidade NOVA, 1169-056 Lisboa, Portugal*
[c]*Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA*
†Electronic supplementary information (ESI) available. See DOI: 10.1039/c5tx00176e

1 Introduction

The non-nucleoside reverse transcriptase inhibitor (NNRTI) neviparine (NVP, **1**, Scheme 1) is the antiretroviral drug most commonly used in developing countries; its low cost and availability as a generic drug explains the widespread use of NVP in these settings, both as a single drug to prevent mother-to-child HIV transmission, and as a component of combination antiretroviral therapy (cART).[1–3] NVP is still a first line choice for initial therapy regimens in developed countries for children younger than 3 years of age who have not been exposed to NVP as part of mother-infant prophylaxis.[4] However, despite its
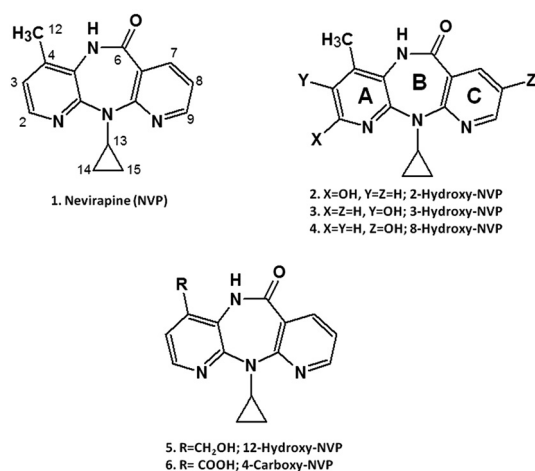
Scheme 1 Structures of nevirapine (NVP, 1) and its Phase I metabolites (2–6).

high efficacy, favorable lipid profile,[5] and suitability for use during pregnancy and breastfeeding,[6,7] NVP therapy is associated with serious and clinically restrictive idiosyncratic toxic events. Severe, life-threatening, and in some cases fatal, liver and skin toxicity have been reported in patients treated with NVP.[8–12]

Several *in vitro* and *in vivo* approaches have been used to address the potential role of reactive metabolites in NVP's toxicity. Cytochrome P450 (CYP)-mediated oxidation, subsequent glucuronidation, and urinary excretion of the glucuronide conjugates represent the primary routes of NVP biotransformation and elimination in humans.[13] Phase I biotransformation of NVP involves CYP-mediated aromatic hydroxylation, yielding the phenolic metabolites 2-, 3-, and 8-hydroxy-NVP[13,14] (2–4, Scheme 1), and C12 oxidation, yielding 4-hydroxymethyl-NVP (12-hydroxy-NVP, 5) and 4-carboxy-NVP (6). Two NVP mercapturates have been identified in the urine of HIV-positive individuals on therapeutic doses of NVP.[15] The major mercapturate, through NVP-C3 (7, Scheme 2), was suggested to be formed by an initial nucleophilic attack of glutathione (GSH) on the epoxide 8, followed by dehydration of the ring-opened intermediate to yield the conjugate 9, which then underwent catabolism to 7. The minor mercapturate, through NVP-C12, was proposed to stem from the GSH attack on a quinone-methide or a sulfate conjugate derived from 5, followed by catabolism. The identification of these Phase II metabolites represented the first evidence of distinct NVP bioactivation pathways, involving either the formation of reactive precursors of phenolic metabolites or the formation of C12-based electrophiles. C12 bioactivation has gained increased support as a major pathway in NVP-induced toxicity.[16–20] In particular, the quinone-methide was proposed to be responsible for NVP-induced liver injury, while 12-sulfoxy-NVP was suggested to be involved in NVP-induced skin rash.[18–20] However, despite considerable efforts in recent years towards the elucidation of the exact mechanisms of NVP-induced toxic outcomes, there are still gaps in our knowledge. Namely, the reasons underlying the higher risk of NVP toxicity in women[21,22] are yet to be identified. Recent reports that both 12-hydroxy-NVP (5) and 3-hydroxy-NVP (3) were found in higher proportions in women[23] suggest that this phenolic NVP metabolite may be an additional factor in the sex-dependent dimorphic profile of NVP toxicity. Moreover, as opposed to 12-hydroxy-NVP, the relative concentration of 2-hydroxy-NVP (2) decreases while the relative concentration of 3-hydroxy-NVP (3) increases when going from a single dose to steady state administration.[24] These observations raise the possibility that 2-hydroxy-NVP may have a role in NVP-induced toxic events arising within the first weeks of treatment,[6] whereas 3-hydroxy-NVP may, to some extent, be involved in chronic NVP-induced toxicity. Considering the key role of covalent adduct formation in drug-induced toxicity, it is pertinent to investigate whether or not oxidative activation of the phenolic NVP metabolites to electrophilic intermediates can result in covalent binding to bionucleophiles under biologically plausible conditions.

We have previously demonstrated the formation of a quinone-imine derivative (10, Scheme 2) upon chemical and enzymatic (lactoperoxidase) oxidation of 2-hydroxy-NVP *in vitro*;[25] the product profile obtained in aqueous media (*e.g.*, the spiro compound 11) was consistent with the transient formation of 10, which underwent prompt nucleophilic attack by water. Should this quinone-imine be formed *in vivo*, its marked electrophilicity suggests that covalent binding with nucleophilic residues in biomacromolecules (*e.g.*, proteins) would likely occur. Moreover, similar to what is reported for other phenolic compounds, phenolic NVP metabolites may undergo alternative oxidative bioactivation to quinone electrophiles (*e.g.*, 12), also capable of reacting with bionucleophiles[26,27] (Scheme 2).

As a further contribution to understand the potential role of phenolic metabolites at the onset of toxic outcomes elicited by NVP, we report herein the formation of a common quinone intermediate upon oxidation of 2- and 3-hydroxy-NVP. The reactivity of the transient NVP-derived quinoid derivatives towards representative bionucleophiles is also addressed.

2 Materials and methods

2.1 Chemicals

NVP‡ was obtained from Cipla (Mumbai, India). L-Amino acids and all other commercially available reagents were acquired from Sigma-Aldrich Química, S.A. (Madrid, Spain), unless specified otherwise, and used as received. Whenever necessary, solvents were purified by standard methods.[28] 2-Hydroxy-NVP (2), 3-hydroxy-NVP (3), and the 1*H*-pyrrole-2,5-dione (13) were synthesized as described in Antunes *et al.*,[25] Grozinger *et al.*,[29] and Antunes *et al.*,[30] respectively. The oxidation reactions of metabolite 2 were performed as described in Antunes *et al.*[25]

---

‡ **Caution:** NVP and its derivatives are potentially carcinogenic. They should be handled with protective clothing in a well-ventilated fume hood.
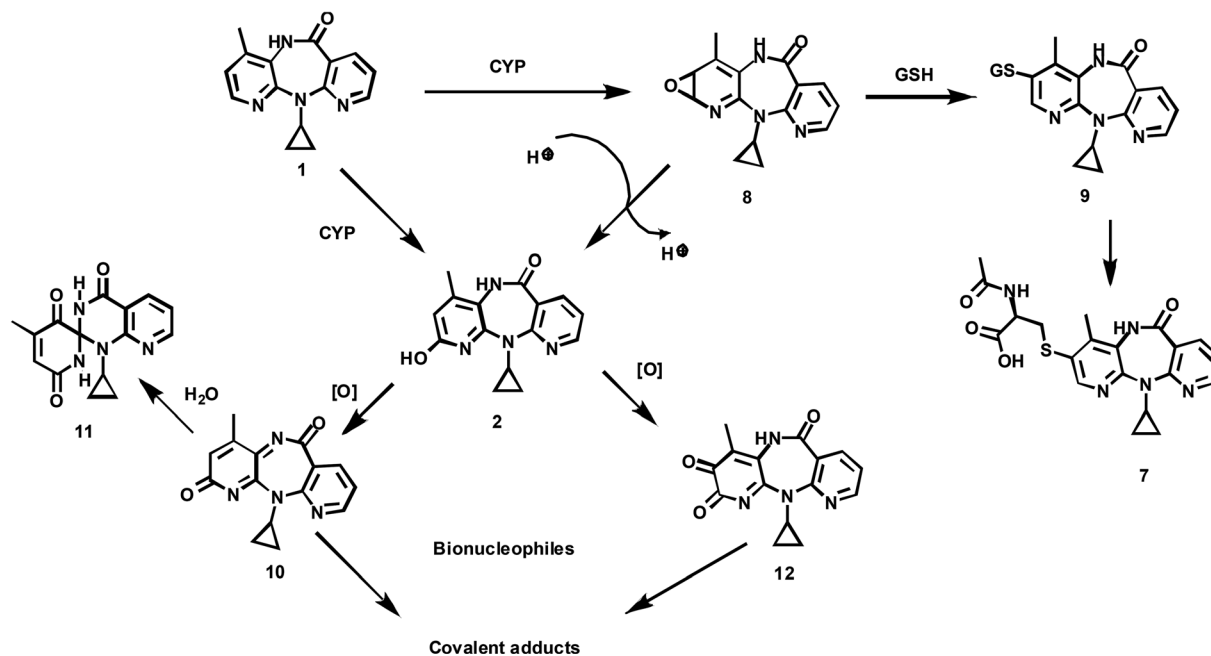
Scheme 2 Proposed pathways for the generation of covalent adducts from 2,3-NVP-epoxide (**8**) and 2-hydroxy-NVP (**2**). CYP, cytochrome P450; GSH, glutathione.

2.2 Instrumentation

2.2.1 HPLC. HPLC analyses were conducted on an Ultimate 3000 Dionex system, consisting of an LPG-3400A quaternary gradient pump and a diode array spectrophotometric detector (Dionex Co., Sunnyvale, CA), and equipped with a Rheodyne model 8125 injector (Rheodyne, Rohnert Park, CA). HPLC analyses were performed with a Luna C18 (2) column (250 mm × 4.6 mm; 5 μm; Phenomenex, Torrance, CA), at a flow rate of 1 mL min^{-1}. Semipreparative HPLC separations were conducted with a Luna C18 (2) column (250 mm × 10 mm; 5 μm; Phenomenex) at a flow rate of 3 mL min^{-1}. A 30 min linear gradient from 5 to 70% acetonitrile in 0.1% aqueous formic acid, followed by a 2 min linear gradient to 100% acetonitrile and an 8 min isocratic elution with acetonitrile, was used in all instances. The UV absorbance was monitored at 254 nm.

2.2.2 Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). LC-ESI-MS/MS analyses were performed with a ProStar 410 autosampler, two 210-LC chromatography pumps, a ProStar 335 diode array detector, and a 500-MS ion trap mass spectrometer, with an ESI ion source (Varian, Inc., Palo Alto, CA). Data acquisition and processing were performed using Varian MS Control 6.9 software. The samples were injected onto the column *via* a Rheodyne injector with a 20 μL loop. Separations were conducted at 30 °C, using a Luna C18 (2) column (150 mm × 2 mm, 3 μm; Phenomenex, Torrance, CA). The mobile phase was delivered at a flow rate of 200 μL min^{-1} using the same solvent gradient described above. The mass spectrometer was

operated in the positive ESI mode; the optimized operating parameters were: ion spray voltage, +5.2 kV; capillary voltage, 80 V; and RF loading, 70%. Nitrogen was used as the nebulizing and drying gas, at pressures of 50 and 30 psi, respectively; the drying gas temperature was 350 °C. MS/MS spectra were obtained with an isolation window of 2 Da, excitation energy values between 0.9 and 1.7 V, and an excitation time of 10 ms.

2.2.3 NMR. ^{1}H NMR spectra were recorded on Bruker Avance III 400 or 500 spectrometers, operating at 400 and 500 MHz, respectively. ^{13}C NMR spectra were recorded on the same instruments, operating at 100.62 and 125.77 MHz, respectively. Chemical shifts are reported in ppm downfield from tetramethylsilane, and coupling constants (J) are reported in Hz; the subscripts *ortho* and *meta*, refer to *ortho* and *meta* couplings, respectively. The presence of labile protons was confirmed by chemical exchange with D$_2$O. Resonance and structural assignments were based on the analysis of coupling patterns, including the ^{13}C–^{1}H coupling profiles obtained in bidimensional heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum coherence (HSQC) experiments, performed with Bruker standard pulse programs.

2.3 Syntheses

2.3.1 General method for the oxidation of 3-hydroxy-NVP (3) with Frémy's salt. A solution of Frémy's salt (0.6 mg, 2.2 μmol) in 100 mM phosphate buffer (50 μL; pH 10, 7.4, or 5) was added to a solution of **3** (0.5 mg, 1.8 μmol) in acetonitrile

(50 μL) and the mixture was stirred overnight at room temperature.

2.3.2 Stabilization of 2,3-NVP-quinone (12) by reduction with sodium cyanoborohydride

2.3.2.1 Quinone obtained by oxidation of 2-hydroxy-NVP (2). A solution of Frémy's salt (60 mg, 223.6 μmol) in 100 mM phosphate buffer (pH 7.4; 2.5 mL) was added to a solution of **2** (50 mg, 177.1 μmol) in acetonitrile (2.5 mL). The resulting mixture was stirred for 30 min at 37 °C, upon which it was treated with sodium cyanoborohydride (50 mg, 795.7 μmol). The reduced quinone (**16**) was isolated by semi-preparative HPLC.

2.3.2.1.1 11-Cyclopropyl-4-methyl-11,11a-dihydro-1*H*-dipyrido-[3,2-*b*:2′,3′-*e*][1,4]diazepine-2,3,6(5*H*)-trione (**16**). Obtained in 13% yield (7 mg); ε_{254}: 455.4 M^{-1} cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 10.53 (1H, bs, NH), 8.66 (1H, bs, NH), 8.42 (1H, bs, H9), 8.08–8.09 (1H, m, H7), 7.07–7.05 (1H, m, H8), 5.45 (1H, s, H11a), 2.77 (1H, bs, H13), 1.65 (3H, s, CH₃), 0.77–0.72 (2H, bs, H14 + H15), 0.52 (1H, bs, H14/H15), 0.29 (1H, bs, H14/H15); ^{13}C NMR (DMSO-d_6): δ C2 not assigned, 172.9 (C3), 165.8 (C6), 157.1 (C10a), 151.8 (C9), 148.3 (C4a), 141.1 (C7), 119.8 (C6a), 117.8 (C8), 115.8 (C4), 73.3 (C11a), 28.9 (C13), 9.2 (C14/C15), 8.0 (C14/C15), 7.5 (CH₃); MS (ESI): m/z 299 [M + H]⁺.

2.3.2.2 Quinone obtained by oxidation of 3-hydroxy-NVP (3). The reduced quinone (**16**) was generated *in situ* from 0.5 mg of **3**, using the procedure described above for the oxidation of **2** and subsequent reduction with sodium cyanoborohydride. The product was identified on the basis of identical HPLC retention time, UV-vis spectrum, and MS/MS fragmentation pattern when compared with **16** obtained from **2**.

2.3.3 Generation of 2,3-NVP-quinone and reaction with bionucleophiles

2.3.3.1 From 2-hydroxy-NVP (2)

2.3.3.1.1 Reaction with ethyl valinate. **Method A.** Ethyl valinate hydrochloride (26 mg, 143.1 μmol) in 100 mM phosphate, pH 7.4 (500 μL), was treated with sodium hydrogen carbonate (12 mg, 143.2 μmol) for 30 min. A solution of **2** (10 mg, 35.4 μmol) in acetonitrile (500 μL) was treated concomitantly with Frémy's salt (12 mg, 44.7 μmol) in 100 mM phosphate buffer, pH 7.4 (500 μL) for 30 min and the mixture was added to the ethyl valinate solution. The resulting mixture was incubated at 37 °C overnight. The reaction was subsequently conducted on a preparative scale, starting from 129 mg of ethyl valinate hydrochloride and 50 mg of **2**. Following overnight incubation at 37 °C the reaction mixture was purified by preparative thin layer chromatography (PTLC) on silica (1/10 methanol/dichloromethane, v/v) yielding **23**.

Ethyl 3-methyl-2-[(4-{[(2-cyclopropylaminopyridin-3-yl)carbonyl]-amino}-3-methyl-2-oxo-2*H*-pyrrol-5-yl)amino]butanoate (**23**). Obtained in 9.6% yield (7 mg); ^{1}H NMR (acetone-d_6): δ 9.50 (1H, bs, NH), 9.16 (1H, bs, NH), 8.32 (1H, dd, J_{ortho} = 4.7, J_{meta} = 1.8, pyridine-H6), 8.17 (1H, bs, NH), 8.03 (1H, dd, J_{ortho} = 7.8, J_{meta} = 1.8, pyridine-H4), 6.69 (1H, dd, J_{ortho} = 7.8, J'_{ortho} = 4.7, pyridine-H5), 4.17–4.09 (3H, m, Val Hα + Val O–C$\underline{H}_2$CH₃),

ca. 2.9 (cyclopropyl-NC$\underline{H}$, obscured by the water resonance), 2.31–2.26 (1H, m, Val isopropyl-C$\underline{H}$), 2.02 (3H, s, CH₃), 1.22 (3H, t, J = 8.5, Val O–CH₂C$\underline{H}_3$), 0.93 (6H, d, J = 6.7, Val isopropyl-C$\underline{H}_3$), 0.79–0.75 (2H, m, cyclopropyl CH + C′H), 0.52–0.48 (2H, m, cyclopropyl CH + C′H); ^{13}C NMR (acetone-d_6): δ 172.4 (pyrr-C2), 170.5 (Val-C=O), 165.8 (NHC=O), 159.3 (pyridine-C2), 153.2 (pyridine-C6), 151.5 (pyrr-C5), 137.1 (pyridine-C4), 136.5 (pyrr-C4), 117.3 (pyrr-C3), 111.7 (pyridine-C5), 108.9 (pyridine-C3), 67.9 (Val-Cα), 60.6 (Val O–C$\underline{H}_2$CH₃), 32.0 (Val isopropyl-C$\underline{H}$), 23.9 (cyclopropyl-NC$\underline{H}$), 18.9 (Val isopropyl-C$\underline{H}_3$), 17.4 (Val isopropyl-C$\underline{H}_3$), 13.8 (Val O–CH₂C$\underline{H}_3$), 10.0 (CH₃), 6.7 (cyclopropyl CH₂ + CH₂); MS (ESI): m/z 436 [MNa]⁺, 414 [MH]⁺.

Method B. Frémy's salt (12 mg, 44.7 μmol) in 100 mM phosphate buffer, pH 7.4 (2 mL) was added to a solution of **2** (10 mg, 35.4 μmol) in acetonitrile (1 mL) and the resulting mixture was stirred for 30 min at 37 °C. Following extraction with dichloromethane (1 mL), the organic layer was dried under a nitrogen stream and redissolved in THF (500 μL). The solution was subsequently added to a solution of ethyl valinate prepared by treating ethyl valinate hydrochloride (26 mg, 143.1 μmol) in 100 mM phosphate buffer, pH 7.4 (500 μL) with sodium hydrogen carbonate (12 mg, 143.1 μmol) for 30 min. The resulting mixture was incubated overnight at 37 °C.

2.3.3.1.2 Reactions with *N*-acetylcysteine and lysine. **Method A.** The quinone **12** was generated from reaction of **2** (10 mg, 35.4 μmol) with Frémy's salt (12 mg, 35.4 μmol), as described above. The reaction mixture was then incubated with *N*-acetylcysteine (35 mg, 214 μmol) or lysine (21 mg, 141 μmol) in 50 mM phosphate buffer, pH 7.4 (500 μL) and the incubation was continued overnight at 37 °C.

Method B. The quinone **12** was prepared from **2** (10 mg, 35.4 μmol) as in method A. After stirring for 30 min at 37 °C, the mixture was extracted with dichloromethane (1 mL), and the organic layer was dried under a nitrogen stream and redissolved in THF (1.0 mL). The solution was subsequently added to a solution of *N*-acetylcysteine (35 mg, 214 μmol) or lysine (21 mg, 141 μmol) in 100 mM phosphate buffer, pH 7.4 (500 μL) and the mixture was incubated overnight at 37 °C.

2.3.3.2 From 3-hydroxy-NVP (3). A solution of ethyl valinate was prepared by treating ethyl valinate hydrochloride (1.3 mg, 7.2 μmol) with sodium hydrogen carbonate (0.6 mg, 7.2 μmol) in 100 mM phosphate buffer, pH 7.4 (5 μL). A solution of **3** (0.5 mg, 1.8 μmol) in acetonitrile (25 μL), was treated concomitantly with a solution of Frémy's salt (0.6 mg, 2.2 μmol) in 100 mM phosphate buffer, pH 7.4 (25 μL) for 30 min, and the mixture was added to the ethyl valinate solution. The resulting mixture was stirred overnight at 37 °C.

2.3.4 Reactions with 2-cyclopropylamino-*N*-(4′-methyl-2′,5′-dioxo-2′,5′-dihydro-1*H*-pyrrol-3′-yl)pyridine-3-carboxamide (13)

2.3.4.1 Susceptibility to hydrolysis and oxidation. A solution of **13** (1.5 mg, 5.2 μmol) in acetonitrile (100 μL) was mixed with 100 mM phosphate buffer (100 μL; pH 5, 7.4, or 10). Similar incubations were performed in the presence of Frémy's salt, as described above for **2** and **3**, and an additional

incubation was performed at pH 7.4 in the presence of sodium cyanoborohydride.

2.3.4.2 Reaction with bionucleophiles

2.3.4.2.1 Reaction with ethyl valinate. A solution of **13** (10 mg, 34.9 μmol) in THF (500 μL) was mixed with a solution of ethyl valinate (from 25 mg of ethyl valinate hydrochloride) in 100 mM phosphate, pH 7.4 (500 μL). The resulting mixture was incubated at 37 °C for 48 h.

2.3.4.2.2 Reaction with *N*-acetylcysteine. A solution of *N*-acetylcysteine (34 mg, 208.3 μmol) in 100 mM phosphate buffer, pH 7.4 (500 μL) was added to a solution of **13** (10 mg, 34.9 μmol) in THF (500 μL). The resulting mixture was stirred for 48 h at 37 °C.

2.4 Enzyme-mediated oxidation of 2-hydroxy-NVP (2)

A solution of **2** (1 mg; 3.54 μmol) in DMSO (40 μL) was added to a solution containing lactoperoxidase (0.4–0.7 U), hydrogen peroxide (20 μL, 1 M solution), and ethyl valinate (6.4 mg, 0.36 μmol) in 100 mM phosphate buffer pH 7.4 (incubation final volume of 1 mL). The resulting mixture was incubated at 37 °C for 30 minutes and then one aliquot (250 μL) was collected into a tube containing acetonitrile (250 μL) and

analyzed by LC-MS/MS. Similar incubations were performed with tyrosinase (14 U), in the absence of hydrogen peroxide, and with myeloperoxidase (5 U) in the presence of KCl (150 mM). In each instance, two control incubations were performed using the same conditions, one in the absence of enzyme and the other in the absence of enzyme and hydrogen peroxide.

3 Results and discussion

3.1 Oxidation of 2-hydroxy-NVP and 3-hydroxy-NVP with Frémy's salt

Our preliminary studies on the chemical oxidation of 2-hydroxy-NVP (**2**) with Frémy's salt[25] consistently indicated the formation of a product, subsequently identified as the 1*H*-pyrrole-2,5-dione derivative **13**[30] (Scheme 3). This product was absent from reaction mixtures obtained upon oxidation of **2** with sodium periodate, suggesting that **13** did not stem from a transient quinone-imine **10**, proposed to be involved in the formation of the spiro product **11**.[25]

To investigate if the phenolic NVP metabolites **2** and **3** could be oxidized to a common reactive intermediate, we
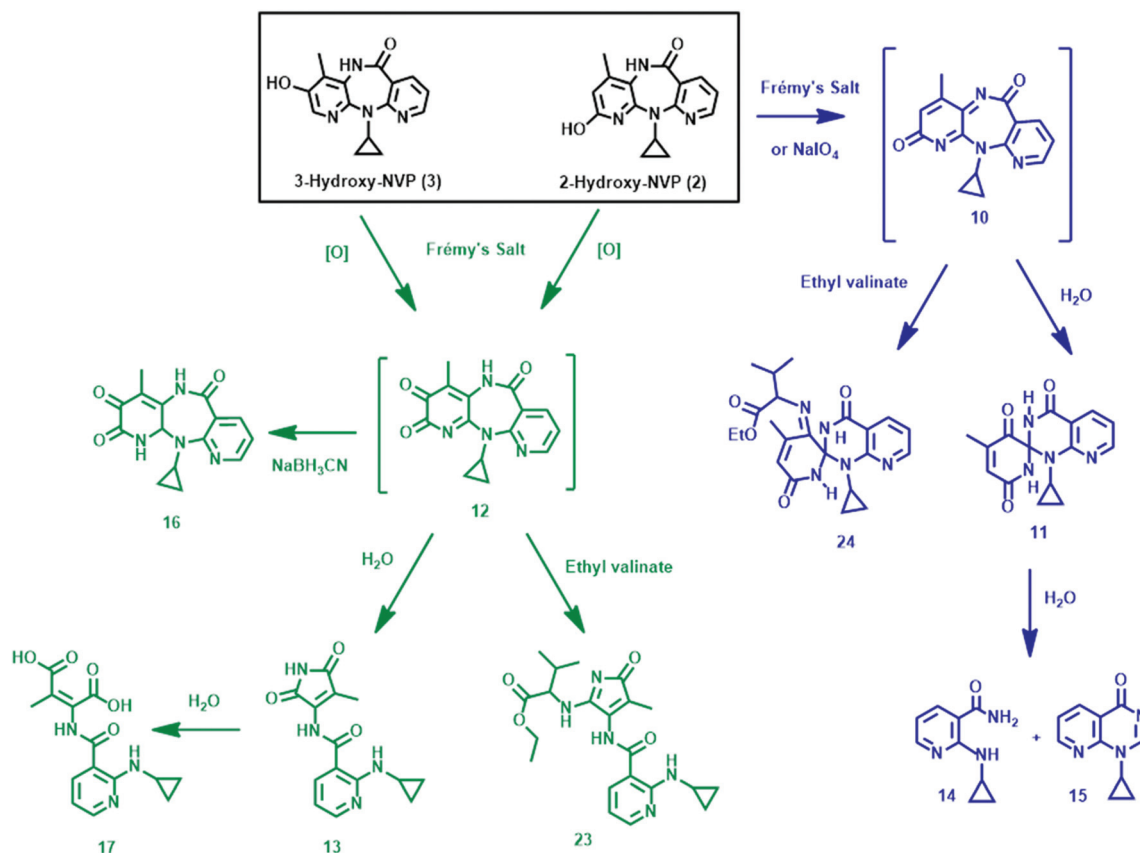
Scheme 3 Proposed oxidative activation pathways of the phenolic metabolites 2-hydroxy-NVP (**2**) and 3-hydroxy-NVP (**3**) showing the products obtained upon oxidation of **2** and **3** with Frémy's salt, under the different experimental conditions used in this study. As indicated in the text, evidence for bioactivation through the quinone-imine **10** and/or the quinone **12** was also obtained in the presence of selected enzymes (lactoperoxidase, myeloperoxidase, or tyrosinase).

studied the oxidation of 3-hydroxy-NVP (**3**) *in vitro*. When **3** was subjected to the same experimental conditions used for the oxidation of **2** with Frémy's salt, the 1*H*-pyrrole-2,5-dione derivative **13** was obtained (Scheme 3); by contrast, neither the spiro product **11** nor its hydrolytic products **14** and **15**, were formed from **3** under these conditions. These results indicate that **2** and **3** share a common oxidized intermediate, different from the quinone-imine **10**. Given that Frémy's salt is frequently employed for the generation of quinones from phenolic derivatives,[31,32] mimicking the one-electron oxidation steps of enzyme-mediated metabolic oxidations,[33–35] the quinone **12** is a plausible common intermediate in the oxidation of the two phenolic NVP metabolites. When the reaction mixtures were analyzed by LC-ESI-MS/MS, either 15 or 30 min after addition of the oxidizing agent, the protonated molecule of **12** (*m/z* 297) was not detected, presumably due to instability of the quinone under the elution conditions used. However, when sodium cyanoborohydride was added 15 or 30 minutes after addition of Frémy's salt the same product (**16**, Fig. 1 and Scheme 3), stemming from reduction of the imine functionality of **12**, was obtained, regardless of the starting phenolic metabolite (**2** or **3**).

The stabilization conferred upon selective reduction of the imino bond allowed the isolation of **16** by semi-preparative HPLC and its subsequent structural characterization by NMR and MS. The ESI-MS spectrum of the stabilized quinone **16** displayed a signal for the protonated molecule at *m/z* 299; MS/MS of this ion yielded a single fragment ion at *m/z* 161, corresponding to the [2-(cyclopropylamino)pyridin-3-yl](oxo)methylium cation, consistent with the assigned structure. The ^{1}H and ^{13}C NMR spectra displayed all of the expected resonances for the NVP ring C (Scheme 1), indicating that this ring was preserved through the oxidation/reduction processes. Moreover, the presence of two exchangeable signals at 10.53 and 8.66 ppm in the ^{1}H NMR spectrum was compatible with the presence of two amide protons in the molecule. The most distinctive feature in the NMR spectra of **16** was an HSQC correlation between a proton singlet at 5.45 ppm and a ^{13}C signal at 73.3 ppm; this resonance was compatible with a tertiary carbon bound to two electron withdrawing nitrogen substituents and entirely consistent with the assigned structure.

The stabilization of 2,3-NVP-quinone (**12**) by reduction with sodium cyanoborohydride allowed the unambiguous confirmation that **12** was the precursor of the 1*H*-pyrrole-2,5-dione derivative **13**. Hence, as shown in Fig. 1, the yield of **13** decreased dramatically upon addition of cyanoborohydride. This is consistent with formation of **13** *via* initial water addition to the imino carbon of **12**. Of note, HPLC-DAD analysis (not shown) indicated that **13** was stable up to 24 h at pH 7.4, in the presence of sodium cyanoborohydride.

We used HPLC-DAD and HPLC-ESI-MS to investigate the degradation of **13** in aqueous solution. Regardless of the presence or absence of Frémy's salt, the major product (**17**; Scheme 3, *m/z* 306 for the protonated molecule in ESI-MS) resulted from stepwise basic hydrolysis of the maleimide to the corresponding dicarboxylate, with no traces of **13** being
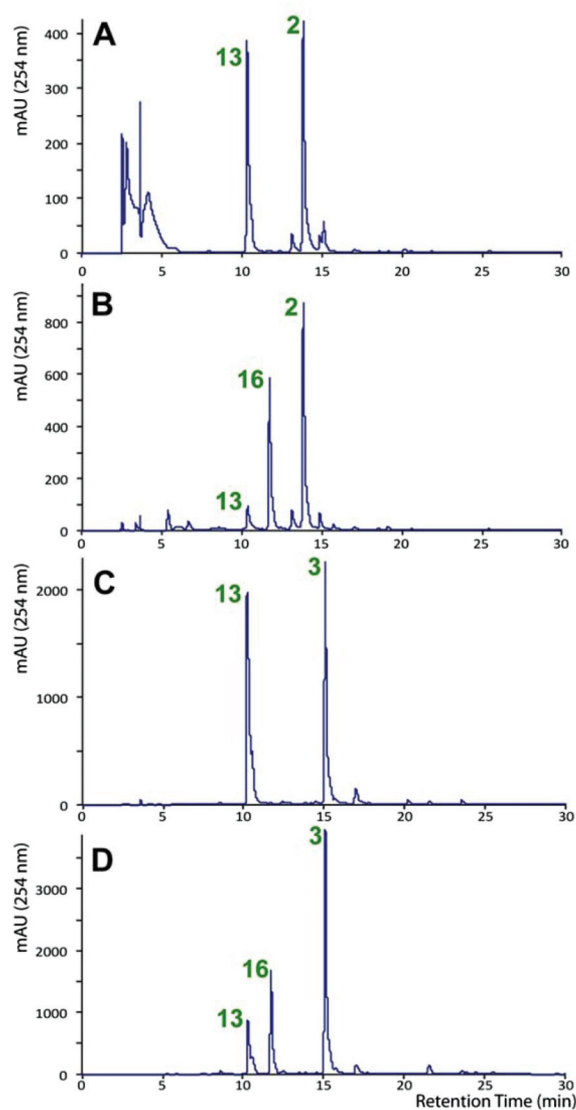

Fig. 1 Representative HPLC-DAD chromatograms, monitored at 254 nm, of (A) a mixture of 2-hydroxy-NVP (**2**) and Frémy's salt at pH 7.4, recorded 30 minutes after adding the reagents; (B) a mixture of 2-hydroxy-NVP (**2**) and Frémy's salt at pH 7.4, following treatment with sodium cyanoborohydride; (C) a mixture of 3-hydroxy-NVP (**3**) and Frémy's salt at pH 7.4, recorded 30 minutes after adding the reagents; (D) a mixture of 3-hydroxy-NVP (**3**) and Frémy's salt at pH 7.4, following treatment with sodium cyanoborohydride. The elution conditions are outlined in the Materials and methods section.

detected after 24 h at pH 10 and no evidence of further oxidative processes (not shown). It is noteworthy that **17** was consistently detected in oxidation reactions of **2** and **3** and that, under the chromatographic conditions used, it exhibited both a UV profile and retention time (not shown) very similar to those of 2-hydroxy-NVP; this coincidence led us to underestimate the extent of oxidation of 2-hydroxy-NVP by Frémy's salt at pH 7.4 and pH 10, in our previous report.[25]

Fig. 2 displays a comparison of the relative percentages of the compounds detected by HPLC-DAD in the oxidation reac-
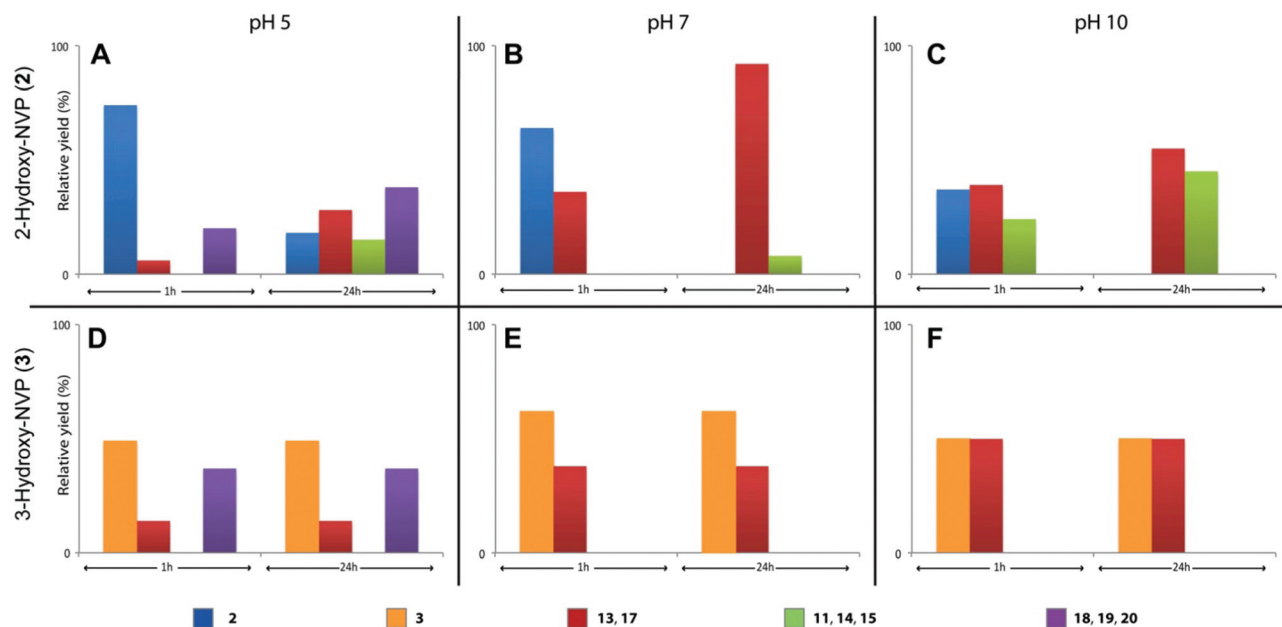
Fig. 2 Relative percentages of the compounds detected by HPLC-DAD in Frémy's salt oxidations of: 2-hydroxy-NVP (**2**) (panels A–C, in aceto-nitrile/100 mM phosphate buffer at pH 5, 7.4, and 10, respectively) and 3-hydroxy-NVP (**3**) (panels D–F, in acetonitrile/100 mM phosphate buffer at pH 5, 7.4, and 10, respectively). The reactions were monitored at 254 nm, 1 h (left) and 24 h (right) after the addition of the oxidant, and the yield was calculated on the basis of the HPLC peak area, corrected for the molar absorptivities of the different products at the monitored wavelength.

tions of 2-hydroxy-NVP (**2**) and 3-hydroxy-NVP (**3**) with Frémy's salt. For simplicity only the overall yields of products from the quinone (**13** and **17**) and quinone-imine pathways (**11**, **14**, and **15**) are shown, rather than the yields of individual compounds. Nonetheless, it should be noted that longer reaction times (24 h) led to an increase in the yields of the final products (**17** and **14**/**15**, respectively).

Several general observations can be drawn upon comparison of the oxidation profiles of **2** and **3**:

(i) Whereas the maleimide **13** and its hydrolysis product **17** were selectively obtained upon oxidation of **3**, a more complex scenario was observed in the oxidation of **2**, with competitive formation of the quinone-imine **10** and subsequent hydrolytic degradation of this intermediate into additional products (Fig. 2 and Scheme 3). Taking into consideration the *meta* positioning of the phenolic hydroxyl and the amide nitrogen, oxidation of **3** cannot lead to the quinone-imine **10**. This explains the selective formation of quinone **12** (subsequently converted into **13**) from **3**.

(ii) Similar to what we reported previously for the formation of hydrolytic products of the quinone-imine **10** from **2**,[25] the oxidation of the two phenolic NVP metabolites, **2** and **3**, into **12** and the subsequent formation of **13** and **17** were considerably affected by the pH. Interestingly, the quinone pathway tended to be favored over the quinone-imine pathway in the oxidation of **2**, particularly at pH 7.4.

(iii) Metabolite **2** was more prone to oxidative degradation than its isomer **3**, which is consistent with the relative susceptibilities of positions 2 and 3 of pyridine rings to oxidation.[36]

(iv) At pH 5, regardless of the phenolic metabolite used, the formation of **13** from the quinone intermediate was clearly less extensive; interestingly, two additional isomeric products, formed under these conditions and displaying signals at *m/z* 315, were identified upon LC-ESI-MS analysis. This observation is consistent with the formation of intermediates **18**, **19**, or **20** (*cf.*, ESI, Scheme S1†). Taken together, these observations support a mechanism involving a transient quinone intermediate **12**, which may undergo water addition to form **21**, followed by ring contraction to **22** *via* a benzil-benzilic acid-type 1,2-rearrangement,[37] and subsequent oxidative decarboxylation to **13** (Scheme S1†).

3.2 Reactions of quinoid NVP derivatives with bionucleophiles

The molecular mechanisms underlying the toxicity of a considerable number of chemical agents have been proposed to involve the formation of quinoid species capable of reacting with bionucleophiles.[26,27] This is illustrated by a few selected examples: (i) *N*-acetyl-*para*-benzoquinone-imine, the electrophilic metabolite responsible for the toxic events induced by paracetamol, presumably due to covalent protein binding when the sacrificial detoxifying agent, glutathione, has been depleted;[38] (ii) the *para*-quinone of the antipsychotic remoxipride, which is thought to be responsible for the aplastic anaemia associated with the parent drug;[39] (iii) dopamine-derived *ortho*-quinones, which react with protein thiols and are likely to be linked with the neurotoxicity induced by these endogenous metabolites;[40] and (iv) 1,2-naphthoquinone, a

metabolite of the environmental pollutant naphthalene, which is thought to affect cell signaling by reacting with cysteinyl residues of proteins.[41] To address the potential toxicological significance of the NVP-derived quinoid intermediates, we investigated the reactions of these metabolites with model amino acids.

Adducts formed by N-terminal valine residues of hemoglobin (Hb) have been applied as biomarkers of drug bioactivation to reactive metabolites and drug–protein modification[42–46] and have been used to study the ability of drug metabolites to undergo protein haptenation.[47] As such, we investigated the ability of 2,3-NVP-quinone (12) and the quinone-imine (10) to react with ethyl valinate, mimicking the N-terminal Hb residue. Towards this goal, we conducted oxidation reactions of 2 and 3 with Frémy's salt at pH 7.4 and after 30 min a solution of ethyl valinate in phosphate buffer (pH 7.4) was added. Under these experimental conditions, the bionucleophile effectively competed with the hydrolysis of 2,3-NVP-quinone (12). This was demonstrated by a marked decrease in the yield of the hydrolysis product 13 upon addition of the bionucleophile, accompanied by the emergence of a new reaction product (cf. ESI, Fig. S2,† for comparison of the HPLC-DAD profiles of the reactions conducted with and without addition of ethyl valinate).

One major adduct (23, Scheme 3) was formed from both 2 and 3 (Fig. 3), which is consistent with 2,3-NVP-quinone (12) being the precursor of this product. The structural assignment of 23 was based on both NMR and MS analyses. Thus, whereas, the ^{1}H and ^{13}C NMR spectra of 23 displayed all expected resonances for the ethyl valinate, NVP ring C and NVP cyclopropyl moieties, only 13 distinct NVP-derived carbons were present in the ^{13}C-NMR spectrum, suggesting that ring contraction was involved in the formation of this adduct. In addition, the methyl protons from the pyrrolidone moiety of 23 (2.02 ppm) had ^{1}H–^{13}C 3-bond HMBC correlations with the pyrrolidone quaternary carbons C2 (172.4 ppm), with a resonance compatible with a carbonyl group, and C4 (136.5 ppm), along with a 2-bond correlation with C3 (117.3 ppm) (Fig. 4); this correlation pattern was remarkably similar to the one we observed for product 13,[30] indicating structural similarity between the two compounds. Moreover, a [1]H–[13]C 3-bond correlation observed between the valine Hα and a quaternary carbon at 151.5 ppm provided definitive proof of connectivity through the ethyl valinate nitrogen and carbon C5 of the pyrrolidone ring of 23 (Fig. 4). Furthermore, the ESI-MS spectrum of this adduct displayed a signal for the protonated molecule at m/z 414 and the corresponding MS/MS spectrum was compatible with the assigned structure (cf. ESI, Scheme S2† for the proposed fragmentation mechanism). Adduct 23 is proposed to be formed by a mechanism similar to the one depicted in Scheme S1† for the formation of maleimide 13, involving nucleophilic attack by the ethyl valinate nitrogen on carbon C11a of 2,3-NVP quinone.

One additional minor product (24), eluting ca. 2 min later than 23 under the conditions used for HPLC-ESI-MS analysis, was detected in the reaction mixtures obtained from 2 and
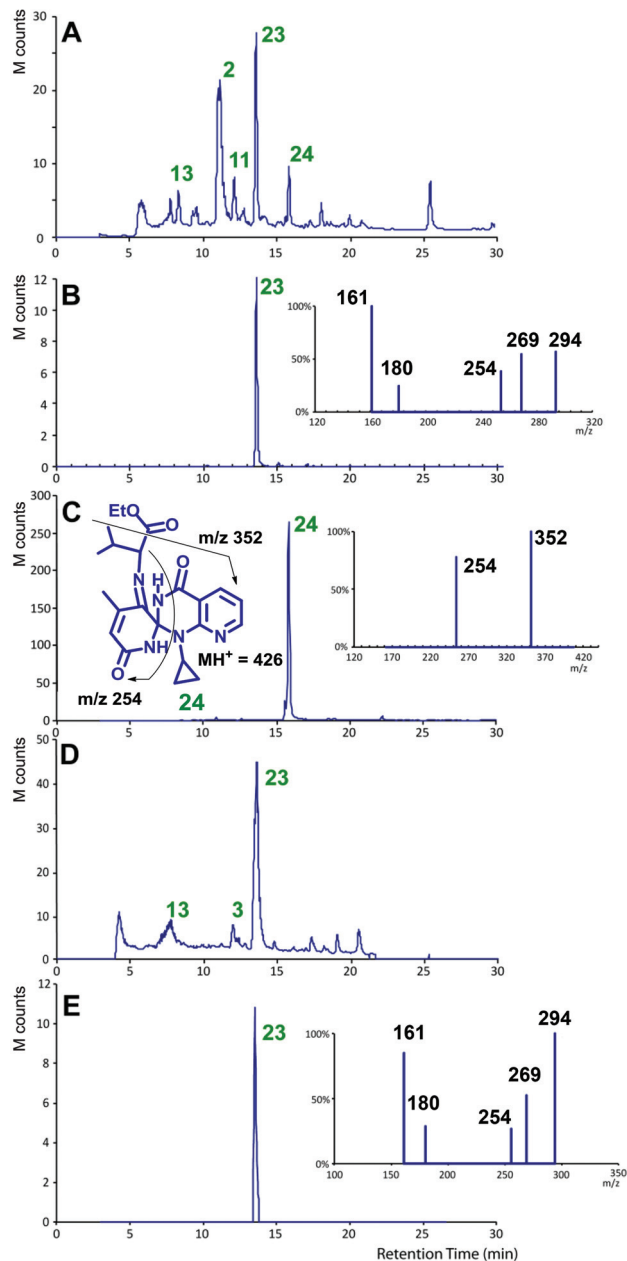

Fig. 3 LC-ESI-MS analyses of the reaction mixture obtained upon oxidation of 2-hydroxy-NVP (2) with Frémy's salt, followed by addition of ethyl valinate: (A) total ion chromatogram; (B) tandem mass chromatogram and mass spectrum of the m/z 414 ion, corresponding to the protonated molecule of adduct 23 (cf. Scheme S2† for the fragmentation mechanisms); (C) tandem mass chromatogram and mass spectrum of the m/z 426 ion, corresponding to the protonated molecule of adduct 24. LC-ESI-MS analyses of the reaction mixture obtained upon oxidation of 3-hydroxy-NVP (3) with Frémy's salt, followed by addition of ethyl valinate: (D) total ion chromatogram; and (E) tandem mass chromatogram and mass spectrum of the m/z 414 ion, corresponding to the protonated molecule of adduct 23. The elution conditions are outlined in the Materials and methods section. Also displayed is the proposed fragmentation pattern of adduct 24.
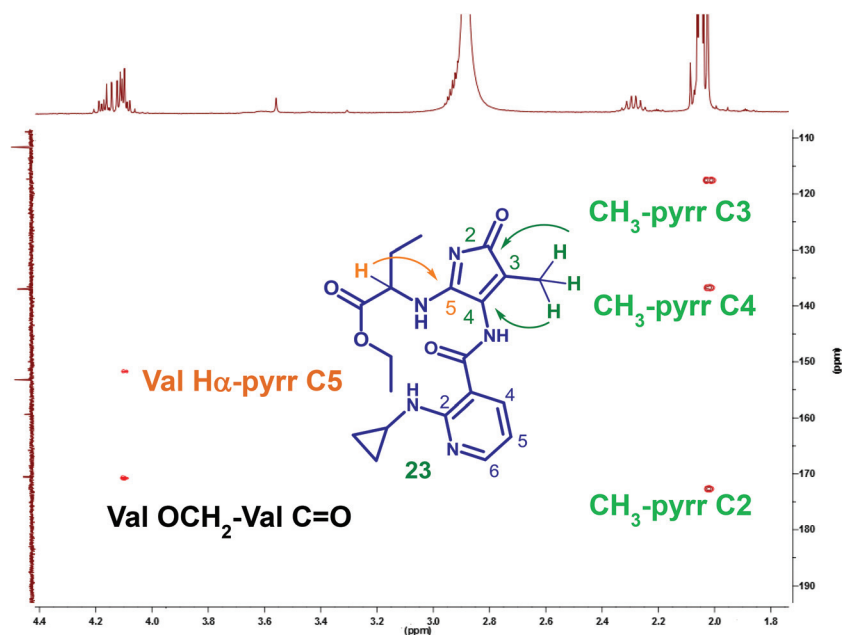
Fig. 4 Expanded region of the $^1H-^{13}C$ HMBC spectrum of adduct **23**, displaying the connectivities between the α proton of ethyl valinate and the pyrrolidone quaternary carbon C5. Also shown are the connectivities between the methyl protons and the pyrrolidone quaternary carbons C2, C3 and C4.

ethyl valinate (Fig. 3). Given that adduct **24** was absent from oxidation reactions of **3** and was formed in considerable lower yield than adduct **23**, it is plausible that it stemmed from a nucleophilic attack to the quinone-imine intermediate **10**; as indicated above, oxidation of **2** *via* the quinone-imine pathway was clearly disfavored compared to the quinone pathway at pH 7.4. Nonetheless, it should be noted that, contrasting with what was observed with **13** and **23**, the emergence of adduct **24** was not accompanied by a marked decrease in the yield of the spiro compound **11**, the hydrolysis product of the quinone-imine **10**. This suggests that ethyl valinate is more efficient at competing with water for the quinone **12** than for the quinone-imine **10**.

Although definite structural characterization of adduct **24** by NMR analysis was precluded by the small amount and low purity of the isolated product, the structure (Fig. 3) was tentatively assigned based on mass spectrometric data. This adduct displayed a signal at m/z 426 in the ESI-MS spectrum, compatible with the protonated molecule from a compound containing an ethyl valinate moiety attached to an NVP-derived moiety with 281 Da. This indicated that adduct **24** did not stem from a ring contraction process (expected from nucleophilic attack to **12**) and was likely to contain an NVP-derived spiro moiety, consistent with a product of nucleophilic attack to the quinone-imine **10**. The fragment ions at m/z 254 and 352 obtained in the tandem mass spectrum are also compatible with the assigned structure (Fig. 3). These data represent the first evidence supporting our initial hypothesis[25] that the intermediate quinone-imine **10** may undergo a nucleophilic attack by bionucleophiles, affording covalent adducts.

LC-ESI-MS analysis of the reaction mixture obtained upon oxidation of **2** with Frémy's salt in the presence of lysine demonstrated that this amino acid is also capable of reacting with quinone **12**. Although the small amount of isolated product precluded its NMR characterization, the ESI-MS spectrum displayed a signal at m/z 415, consistent with the protonated molecule of a lysine analogue of **23**. The identity of this adduct (**25**) was further corroborated by MS/MS analysis, which afforded a fragmentation pattern fully compatible with the assigned structure (*cf.* ESI, Fig. S3†). Taken together, the results from the reactions with ethyl valinate and lysine represent conclusive evidence of the ability of 2,3-NVP-quinone (**12**) to be trapped by nitrogen-based bionucleophiles.

In general, reactive quinones and quinone-imines are especially prone to Michael addition by highly nucleophilic sulfhydryl groups. Hence, adduct formation with glutathione and with cysteine residues of proteins is a major mechanism of both detoxification and toxicity of these electrophilic species.[26,27] As such, we also investigated the ability of *N*-acetylcysteine (NAC) to trap the quinoid intermediates **10** and **12**.

In addition to experimental conditions identical to those used for the modification of ethyl valinate (in which NAC was added directly to the reaction mixture, 30 min after the addition of Frémy's salt), an alternative procedure was used to avoid Frémy's salt-promoted NAC dimerization. The procedure involved extraction of the electrophilic intermediates with dichloromethane, 30 min after starting the oxidation of **2**. The method was first tested using ethyl valinate as the nucleophile, leading to the same reaction products detected using the experimental approach described above.

Regardless of the experimental conditions, the only product detected in the reactions with NAC (**26**, *cf.* ESI, Fig. S4†), stemmed from NAC addition to product **13**. This was confirmed by comparison of HPLC retention time and mass spectrum with those of the products obtained in the direct reaction of **13** with NAC. Hence, contrasting with the classic reactivity of quinoid intermediates toward sulfur nucleophiles, no evidence was obtained for reaction of the sulfhydryl group of NAC with **10** or **12**. It is plausible that an initial nucleophilic attack by NAC on either the quinone-imine **10** or the quinone **12** occurred reversibly,[48] due to the higher ability of sulfhydryl as a leaving group when compared with amine or amide nitrogens (*cf.* ESI, Scheme S3,† for a mechanistic interpretation). Considering that glutathione conjugation of quinoid derivatives represents a typical elimination process for this class of electrophilic metabolites, the lack of productive reaction of 2,3-NVP-quinone (**12**) and the quinone-imine **10** with sulfhydryl groups is expected to hamper the elimination of these intermediates. Moreover, although the biological significance of the maleimide **13** remains to be established, these results suggest that, if formed *in vivo*, it may be trapped by sulfhydryl groups of cysteine residues in proteins. Interestingly, no reaction was detected when **13** was incubated with ethyl valinate, implying selectivity towards sulfhydryl nucleophiles.

3.3 Enzyme-mediated generation of quinoid NVP derivatives

The LC-ESI-MS detection of the spiro product **11** upon incubation of 2-hydroxy-NVP (**2**) with lactoperoxidase, in the presence of hydrogen peroxide, represented the first evidence for the occurrence of oxidative bioactivation of **2** to the electrophilic quinone-imine **10**.[25] However, the formation of the 2,3-NVP-quinone (**12**) under biologically plausible conditions, and the ability of **10** and **12** to be trapped by bionucleophiles remained to be elucidated.

To obtain further insight into the potential biological significance of these oxidative bioactivation pathways, we incubated the phenolic NVP metabolite **2** with several peroxidases (lactoperoxidase, myeloperoxidase, and tyrosinase) in the presence of ethyl valinate and investigated the formation of covalent adducts **23** and **24** by LC-ESI-MS/MS. In addition, the formation of maleimide **13** (the hydrolysis product of quinone **12**) along with the hydrolysis products of quinone-imine **10**, the spiro product **11** and its hydrolytic product **15**, was also investigated. The choice of these enzymes was based on their ability to mediate the oxidation of a considerable number of phenolic substrates to quinoid species that are of crucial relevance to the toxicity induced by several toxicants. For instance, myeloperoxidase, a heme protein enzyme that is present in high concentrations in the neutrophils of blood and bone, is responsible for the ultimate activation of some phenolic metabolites of benzene to catechols and quinones, in the presence of hydrogen peroxide as a cofactor.[49] This bioactivation route is thought to account, in part, for the carcinogenicity of benzene and the numerous cases of benzene-induced leukemia.[50] Tyrosinase is a copper-containing enzyme that

mediates the melanogenesis in human skin, involving the formation of catechols and quinones. The exacerbation of dopamine toxicity *via* tyrosinase-induced formation of quinones is a well-known event.[51] Lactoperoxidase, another member of the heme peroxidase family of enzymes, which is secreted by mammary, salivary, and other mucosal glands, mediates the oxidation of the phenolic ring of estradiol, a conversion that may be involved in breast carcinogenesis.[52,53]

Interestingly, while all tested enzymes had the ability to mediate the oxidation of **2**, distinct selectivities were observed, as evidenced by the considerably different product profiles obtained for each enzyme-mediated oxidation. Based upon identical retention times, detection of the protonated molecules (*m/z* 414 and 287), and MS/MS fragmentation patterns, as compared with the products from Frémy's salt oxidation, we obtained clear evidence for the formation of the covalent adduct **23** and maleimide **13**, in the incubation of **2** with tyrosinase. This suggests that this enzyme selectively catalyzes the oxidation of **2** into 2,3-NVP-quinone (**12**). By contrast, and in accordance with our preliminary results, lactoperoxidase selectively mediated the oxidation of **2** into its quinone-imine intermediate **10**, as evidenced by the detection of the protonated molecules of adduct **24** (*m/z* 426) and of the hydrolysis products **11** and **15** (*m/z* 299 and 188, respectively).

The product profile observed upon Frémy's salt oxidation was replicated in the myeloperoxidase-mediated oxidation, which demonstrates the ability of this enzyme to oxidize **2** into its two quinoid derivatives, the quinone-imine **10** and the quinone **12**. These results represent not only clear evidence that **2** can be bioactivated to its quinoid derivatives by a variety of metabolizing enzymes but also that these electrophilic species can be efficiently trapped by nucleophiles under biomimetic conditions. Whereas conclusive evidence for the toxicological relevance of this oxidative bioactivation can only be achieved upon analysis of clinical samples, these data support our initial hypothesis that the metabolic activation of phenolic NVP metabolites to **10** and **12** is plausible *in vivo*, and could be a factor in some of the toxic events associated with NVP administration.

Considering the presence of high concentrations of myeloperoxidase in neutrophils, and the fact that hepatic neutrophils have been suggested to contribute significantly to the pathogenesis of chemically-induced liver injury,[54] the potential involvement of myeloperoxidase-mediated bioactivation of phenolic NVP metabolites in the hepatotoxicity induced by the parent drug cannot be discounted. Indeed, drug-induced toxic events are frequently mediated by more than one bioactivation pathway. Thus, the considerable increase of myeloperoxidase activity observed in damaged liver, due to the presence of pathologically invasive neutrophils,[55] may imply that even if NVP-induced liver toxicity is initiated by a quinone-methide derived from 12-hydroxy-NVP (**5**) or from direct NVP oxidation,[18] the bioactivation of **2** and/or **3** could contribute to an exacerbation of this toxic effect. Likewise, taking into consideration that the production of myeloperoxidase in the skin,

probably due to pathologically invasive or locally activated macrophages and neutrophils, was suggested to occur in patients with drug-induced toxic epidermal necrolysis,[56] an exacerbation of skin toxicity *via* myeloperoxidase-mediated bioactivation of the phenolic NVP metabolites is conceivable. Also noteworthy is the fact that the myeloperoxidase activity in neutrophils was observed to be higher in women compared with men.[57] This is in agreement with the increased risk for NVP-induced skin toxicity in women, which can reach a 10% incidence and is about 7-fold higher than in men, when adjusted for age and baseline CD4 cell count.[21] Moreover, while the sulfate metabolite derived from **5** was proposed to play a crucial role in NVP-induced toxic events in the skin, the fact that tyrosinase is essential to melanogenesis in human skin cannot be disregarded when considering the potential bioactivation of phenolic metabolites as a risk factor. Furthermore, taking into consideration that NVP is administered concurrently with breastfeeding and the fact that NVP readily enters breast milk, the lactoperoxidase-mediated oxidation of **2** into its quinone-imine **10** could also play a role in NVP-induced toxic events in the perinatal setting.[58]

The fact that the phenolic NVP metabolites **2** and **3** are more extensively glucuronidated than **5** has been argued to discount their potential significance to the toxic events induced by NVP. While the free (non-conjugated) metabolite concentrations in plasma follow the order **5** > **2** > **3**,[23,24] the total (free plus glucuronide) concentrations follow the order **3** > **5** > **2**.[59,60] This implies that any factor affecting the extent of glucuronidation can contribute to increase significantly the systemic exposure to **3**. The fact that women have lower UDP glucuronosyltransferase activity[61,62] suggests that they may be less efficient than men in detoxifying phenolic metabolites, which may contribute to the higher risk for NVP skin toxicity in women. As noted previously, higher concentrations of **3** have been found in women compared to men.[23] Moreover, whereas the specific isoforms of UDP-glucuronosyltransferase involved in the detoxification of NVP metabolites remain to be elucidated, polymorphisms of these enzymes can have a significant impact on the plasmatic levels of **3** and **2**, similar to what is observed with other drugs.[63] One additional consideration when comparing the relative significance of the reactive NVP quinone-methide and/or 12-sulfoxy-NVP metabolites with the electrophiles derived from phenolic metabolites, is their distinct ability to react with sulfhydryl groups. Indeed, while the former readily undergo GSH conjugation,[15] our current work demonstrates the inability of the quinoid derivatives **10** and **12** to react in a similar manner.

4　Conclusions

The present manuscript provides the first report on the ability of two phenolic NVP metabolites, 2- and 3-hydroxy-NVP, to form a common electrophilic intermediate upon oxidation with Frémy's salt. The transient formation of the 2,3-NVP-quinone (**12**) was unambiguously proven upon stabilization by reduction, allowing full NMR and MS characterization. This work provided additional evidence for the existence of two distinct oxidative activation pathways for the phenolic metabolite 2-hydroxy-NVP (**2**), involving the transient formation of the quinone **12** and the quinone-imine **10**. In contrast, the phenolic metabolite 3-hydroxy-NVP (**3**) was selectively activated to quinone **12**.

One additional feature of the current work was to provide experimental evidence for the ability of nitrogen-based bionucleophiles to compete with the hydrolysis of the quinone intermediate **12**. The identification and full structural characterization of adduct **23**, stemming from nucleophilic attack of the model amino acid ethyl valinate on quinone **12**, together with the MS evidence for the formation of its lysine analogue **25**, suggest the possibility of reaction of this electrophilic intermediate with nitrogen-based bionucleophiles (*e.g.*, lysine residues of proteins) *in vivo*. Although the competition with hydrolysis of intermediate **10** was not so efficient, as compared with **12**, the detection of adduct **24** with ethyl valinate in reaction mixtures generated from **2** suggests that quinone-imine **10** can also be trapped by nitrogen-based bionucleophiles. Moreover, the detection of these adducts upon incubation of **2** with several peroxidases (tyrosinase, myeloperoxidase, and lactoperoxidase) in the presence of ethyl valinate represents preliminary evidence supporting the hypothesis that transient formation of intermediates **10** and **12** is plausible *in vivo*. Given that formation of covalent adducts with proteins is a frequent mechanism at the onset of drug-induced toxicity, these quinoid intermediates may have a role in the toxic events associated with NVP.

Although clarification of the role of oxidative bioactivation of phenolic metabolites in NVP toxicity still requires much effort in the analysis of clinical samples, the potential toxicological significance of the quinoid intermediates **10** and **12** is further supported by the observed lack of reactivity toward NAC. Taking into consideration that glutathione conjugation is the major detoxification pathway of this class of electrophilic intermediates, the lack of efficient reaction of both **10** and **12** with sulfhydryl groups is expected to hamper the detoxification of these electrophilic intermediates, if formed *in vivo*, thereby increasing their availability to react with nitrogen-based bionucleophiles.

A common trait of all products identified in this study was the considerable structural degradation of the parent drug, leading to a mass increment inconsistent with that expected from direct oxidation alone. This characteristic stresses the relevance of conducting the current *in vitro* approach, since the identification *in vivo* of products from NVP-derived quinoid species would be highly improbable without this prior knowledge.

Conflict of interest

None to declare.

Acknowledgements

Notes and references

1 E. Marseille, J. G. Kahn, F. Mmiro, L. Guay, P. Musoke, M. G. Fowler and J. B. Jackson, *Lancet*, 1999, **354**, 803–809.

2 M. Lallemant, G. Jourdain, S. Le Coeur, J. Y. Mary, N. Ngo-Giang-Huong, S. Koetsawang, S. Kanshana, K. McIntosh and V. Thaineua for the Perinatal HIV Prevention Trial (Thailand) Investigators, *N. Engl. J. Med.*, 2004, **351**, 217–228.

3 S. Lockman, R. L. Shapiro, L. M. Smeaton, C. Wester, I. Thior, L. Stevens, F. Chand, J. Makhema, C. Moffat, A. Asmelash, P. Ndase, P. Arimi, E. van Widenfelt, L. Mazhani, V. Novitsky, S. Lagakos and M. Essex, *N. Engl. J. Med.*, 2007, **356**, 135–147.

4 Panel on Antiretroviral Therapy and Medical Management of HIV-Infected Children. Guidelines for the use of antiretroviral agents in pediatric HIV infection, Available at http://aidsinfo.nih.gov/ContentFiles/PediatricGuidelines.pdf (last updated March 5, 2015; accessed May 26, 2015).

5 L. Ruiz, E. Negredo, P. Domingo, R. Paredes, E. Francia, M. Balagué, S. Gel, A. Bonjoch, C. R. Fumaz, S. Johnston, J. Romeu, J. Lange, B. Clotet and the Spanish Lipodystrophy Group, *J. Acquired Immune Defic. Syndr.*, 2001, **27**, 229–236.

6 Panel on Treatment of HIV-Infected Pregnant Women and Prevention of Perinatal Transmission. Recommendations for use of antiretroviral drugs in pregnant HIV-1-infected women for maternal health and interventions to reduce perinatal HIV transmission in the United States. Available at http://aidsinfo.nih.gov/contentfiles/lvguidelines/PerinatalGL.pdf (last updated August 6, 2015; accessed September 3, 2015).

7 T. Horvath, B. C. Madi, I. M. Iuppa, G. E. Kennedy, G. W. Rutherford and J. S. Read, *Cochrane Database Syst. Rev.*, 2009, CD006734.

8 R. B. Pollard, P. Robinson and K. Dransfield, *Clin. Ther.*, 1998, **20**, 1071–1092.

9 K.J. Warren, D. E. Boxwell, N. Y. Kim and B. A. Drolet, *Lancet*, 1998, **351**, 567.

10 J.-P. Fagot, M. Mockenhaupt, J.-N. Bouwes-Bavinck, L. Naldi, C. Viboud and J.-C. Roujeau for the EuroSCAR study group, *AIDS*, 2001, **15**, 1843–1848.

11 I. Sanne, H. Mommeja-Marin, J. Hinkle, J. A. Bartlett, M. M. Lederman, G. Maartens, C. Wakeford, A. Shaw, J. Quinn, R. G. Gish and F. Rousseau, *J. Infect. Dis.*, 2005, **191**, 825–829.

12 M. S. Baylor and R. Johann-Liang, *J. Acquired Immune Defic. Syndr.*, 2004, **35**, 538–539.

13 P. Riska, M. Lamson, T. MacGregor, J. Sabo, S. Hattox, J. Pav and J. Keirns, *Drug Metab. Dispos.*, 1999, **27**, 895–901.

14 D. A. Erickson, G. Mather, W. F. Trager, R. H. Levy and J. J. Keirns, *Drug Metab. Dispos.*, 1999, **27**, 1488–1495.

15 A. Srivastava, L.-Y. Lian, J. L. Maggs, M. Chaponda, M. Pirmohamed, D. P. Williams and B. K. Park, *Drug Metab. Dispos.*, 2010, **38**, 122–132.

16 J. Chen, B. M. Mannargudi, L. Xu and J. Uetrecht, *Chem. Res. Toxicol.*, 2008, **21**, 1862–1870.

17 M. Popovic, J. L. Caswell, B. Mannargudi, J. M. Shenton and J. P. Uetrecht, *Chem. Res. Toxicol.*, 2006, **19**, 1205–1214.

18 A. M. Sharma, Y. Li, M. Novalen, M. A. Hayes and J. Uetrecht, *Chem. Res. Toxicol.*, 2012, **25**, 1708–1719.

19 A. M. Sharma, K. Klarskov and J. Uetrecht, *Chem. Res. Toxicol.*, 2013, **26**, 410–421.

20 A. M. Sharma, M. Novalen, T. Tanino and J. P. Uetrecht, *Chem. Res. Toxicol.*, 2013, **26**, 817–827.

21 S. J. Bersoff-Matcha, W. C. Miller, J. A. Aberg, C. van der Horst, H. J. Hamrick Jr., W. G. Powderly and L. M. Mundy, *Clin. Infect. Dis.*, 2001, **32**, 124–129.

22 A. Antinori, F. Baldini, E. Girardi, A. Cingolani, M. Zaccarelli, S. Di Giambenedetto, A. Barracchini, P. De Longis, R. Murri, V. Tozzi, A. Ammassari, M. G. Rizzo, G. Ippolito and A. De Luca, *AIDS*, 2001, **15**, 1579–1581.

23 A. T. Marinho, P. M. Rodrigues, U. Caixas, A. M. M. Antunes, T. Branco, S. G. Harjivan, M. M. Marques, E. C. Monteiro and S. A. Pereira, *J. Antimicrob. Chemother.*, 2014, **69**, 476–482.

24 P. Fan-Havard, Z. Liu, M. Chou, Y. Ling, A. Barrail-Tran, D. W. Haas, A.-M. Taburet and the ANRS12154 Study Group, *Antimicrob. Agents Chemother.*, 2013, **57**, 2154–2160.

25 A. M. M. Antunes, D. A. Novais, J. L. Ferreira da Silva, P. P. Santos, M. C. Oliveira, F. A. Beland and M. M. Marques, *Org. Biomol. Chem.*, 2011, **9**, 7822–7835.

26 J. L. Bolton, M. A. Trush, T. M. Penning, G. Dryhurst and T. J. Monks, *Chem. Res. Toxicol.*, 2000, **13**, 135–160.

27 T. J. Monks and D. C. Jones, *Curr. Drug Metab.*, 2002, **3**, 425–438.

28 D. D. Perrin and W. L. F. Armarego, *Purification of Laboratory Chemicals*, Pergamon Press, Oxford, U. K., 3rd edn, 1988, pp. 1–391.

29 K. G. Grozinger, D. P. Byrne, L. J. Nummy, M. D. Ridges and A. Salvagno, *J. Heterocycl. Chem.*, 2000, **37**, 229–239.

30 A. M. M. Antunes, M. Sidarus, D. A. Novais, S. G. Harjivan, P. P. Santos, J. L. Ferreira da Silva, F. A. Beland and M. M. Marques, *Molecules*, 2012, **17**, 2616–2627.

31 J. G. Marrero, L. San Andrés and J. G. Luis, *Chem. Pharm. Bull.*, 2005, **53**, 1524–1529.

32 J. M. Saá, J. Morey and C. Rubido, *J. Org. Chem.*, 1986, **51**, 4471–4473.

33 H. Zimmer, D. C. Lankin and S. W. Horgan, *Chem. Rev.*, 1971, **71**, 229–246.

34 J. Zielonka, H. Zhao, Y. Xu and B. Kalyanaraman, *Free Radicals Biol. Med.*, 2005, **39**, 853–863.

35 L. Shen, E. Pisha, Z. Huang, J. M. Pezzuto, E. Krol, Z. Alam, R. B. van Breemen and J. L. Bolton, *Carcinogenesis*, 1997, **18**, 1093–1101.

36 T. L. Gilchrist, *Heterocyclic Chemistry*, Addison Wesley Longman Ltd, Essex, U. K., 3rd edn, 1997, pp. 125–158.

37 K. Bowden and W. M. F. Fabian, *J. Phys. Org. Chem.*, 2001, **14**, 794–796.

38 L. P. James, P. R. Mayeux and J. A. Hinson, *Drug Metab. Dispos.*, 2003, **31**, 1499–1506.

39 J. C. L. Erve, M. A. Svensson, H. von Euler-Chelpin and E. Klasson-Wehler, *Chem. Res. Toxicol.*, 2004, **17**, 564–571.

40 M. Asanuma, I. Miyazaki and N. Ogawa, *Neurotoxic. Res.*, 2003, **5**, 165–176.

41 Y. Kumagai, Y. Shinkai, T. Miura and A. K. Cho, *Annu. Rev. Pharmacol. Toxicol.*, 2012, **52**, 221–247.

42 G. Boysen, N. I. Georgieva, P. B. Upton, V. E. Walker and J. A. Swenberg, *Chem.-Biol. Interact.*, 2007, **166**, 84–92.

43 S. Chevolleau, C. Jacques, C. Canlet, J. Tulliez and L. Debrauwer, *J. Chromatogr. A*, 2007, **1167**, 125–134.

44 C. Charneira, N. M. Grilo, S. A. Pereira, A. L. A. Godinho, E. C. Monteiro, M. M. Marques and A. M. M. Antunes, *Br. J. Pharmacol.*, 2012, **167**, 1353–1361.

45 U. Caixas, A. M. M. Antunes, A. T. Marinho, A. L. A. Godinho, N. M. Grilo, M. M. Marques, M. C. Oliveira, T. Branco, E. C. Monteiro and S. A. Pereira, *Toxicology*, 2012, **301**, 33–39.

46 N. M. Grilo, A. M. M. Antunes, U. Caixas, A. T. Marinho, C. Charneira, M. C. Oliveira, E. C. Monteiro, M. M. Marques and S. A. Pereira, *Toxicol. Lett.*, 2013, **219**, 59–64.

47 M. Törnqvist, C. Fred, J. Haglund, H. Helleberg, B. Paulsson and P. Rydberg, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2002, **778**, 279–308.

48 S. Zheng, Y. R. Santosh Laxmi, E. David, A. T. Dinkova-Kostova, K. H. Shiavoni, Y. Ren, Y. Zheng, I. Trevino, R. Bumeister, I. Ojima, W. C. Wigley, J. B. Bliska, D. F. Mierke and T. Honda, *J. Med. Chem.*, 2012, **55**, 4837–4846.

49 V. V. Subrahmanyam, P. Kolachana and M. T. Smith, *Arch. Biochem. Biophys.*, 1991, **286**, 76–84.

50 V. Wiwanitkit, S. Soogarun and J. Suwansaksri, *Leuk. Lymphoma*, 2004, **45**, 1643–1645.

51 E. Greggio, E. Bergantino, D. Carter, R. Ahmad, G.-E. Costin, V. J. Hearing, J. Clarimon, A. Singleton, J. Eerola, O. Hellström, P. J. Tienari, D. W. Miller, A. Beilina, L. Bubacco and M. R. Cookson, *J. Neurochem.*, 2005, **93**, 246–256.

52 H. J. Sipe Jr., S. J. Jordan, P. M. Hanna and R. P. Mason, *Carcinogenesis*, 1994, **15**, 2637–2643.

53 E. M. Ghibaudi, E. Laurenti, P. Beltramo and R. P. Ferrari, *Redox Rep.*, 2000, **5**, 229–235.

54 H. Jaeschke, C. W. Smith, M. G. Clemens, P. E. Ganey and R. A. Roth, *Toxicol. Appl. Pharmacol.*, 1996, **139**, 213–226.

55 A. Amanzada, I. A. Malik, M. Nischwitz, S. Sultan, N. Naz and G. Ramadori, *Histochem. Cell Biol.*, 2011, **135**, 305–315.

56 P. Paquet, D. De Groote and G. E. Piérard, *Dermatology*, 2010, **220**, 201–207.

57 A. Rutgers, P. Heeringa, J. E. H. M. Giesen, R. T. Theunissen, H. Jacobs and J. W. C. Tervaert, *Br. J. Haematol.*, 2003, **123**, 536–538.

58 M. Mirochnick, D. F. Clarke and A. Dorenbaum, *Clin. Pharmacokinet.*, 2000, **39**, 281–293.

59 L. S. Rowland, T. R. MacGregor, S. J. Campbell, R. Jenkins, A. B. Pearsall and J. P. Morris, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2007, **856**, 252–260.

60 A. M. Cammett, T. R. MacGregor, J. M. Wruck, F. Felizarta, P. Miailhes, J. Mallolas and P. J. Piliero, *Antimicrob. Agents Chemother.*, 2009, **53**, 4147–4152.

61 C. J. Gallagher, R. M. Balliet, D. Sun, G. Chen and P. Lazarus, *Drug Metab. Dispos.*, 2010, **38**, 2204–2209.

62 G. D. Anderson, *Int. Rev. Neurobiol.*, 2008, **83**, 1–10.

63 Y. Maruo, M. Iwai, A. Mori, H. Sato and Y. Takeuchi, *Curr. Drug Metab.*, 2005, **6**, 91–99.