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Short communication

The phenolic metabolites of the anti-HIV drug efavirenz: Evidence for distinct reactivities upon oxidation with Frémy's salt

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ABSTRACT

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor administered as first line treatment against HIV-1. The major drawbacks of EFV therapy are neurotoxicity and hepatotoxicity, which may result from bioactivation to reactive metabolites capable of reacting with bionucleophiles. We investigated the *in vitro* oxidation of the phenolic EFV metabolites, 7-hydroxy-efavirenz (7-OH-EFV) and 8-hydroxy-efavirenz (8-OH-EFV), with Frémy's salt. A quinoline derivative, 6-chloro-2-cyclopropyl-4-(trifluoromethyl)quinolin-7-ol, presumably stemming from a radical rearrangement, was selectively obtained from 7-OH-EFV in 10% yield. In contrast, when subjected to the same oxidation conditions, 8-OH-EFV was considerably more prone to oxidative degradation and yielded multiple products. Among these, a quinone–imine derivative was tentatively identified upon LC–ESI–MS/MS analysis of the reaction mixture. These observations demonstrate a remarkable difference in the reactivities of the two phenolic EFV metabolites under oxidative conditions. Moreover, taking into consideration the toxicological significance of quinone–imine derivatives, these findings may explain earlier reports that 8-OH-EFV is a more potent toxicant than 7-OH-EFV in model test systems.

1. Introduction

Efavirenz (EFV, (*S*)-6-chloro-4-(cyclopropylethynyl)-4-(trifluoromethyl)-1,4-dihydro-2*H*-3,1-benzoxazin-2-one, **1**, Scheme 1) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) administered as first-line treatment against HIV [1]. Despite its efficacy, a major limitation of EFV use is its association with clinically restrictive neurotoxic and hepatotoxic events [2–5]. EFV is metabolized by cytochrome P450 (CYP), undergoing primary oxidation on the aromatic ring to the phenolic products 8-hydroxy-efavirenz (8-OH-EFV, **2**, Scheme 1) (major) and 7-hydroxy-efavirenz (7-OH-EFV, **3**) (minor) and secondary oxidation on the cyclopropane ring (at C14) to 8,14-dihydroxy-efavirenz (8,14-diOH-EFV, **4**) [6].

Whereas the molecular mechanisms underlying EFV-induced toxicity remain to be elucidated, an involvement of the phenolic metabolite 8-OH-EFV (**2**) in the onset of the toxic events elicited by the parent drug is consistent with results from *in vitro* studies. The first evidence was provided in primary human hepatocyte cultures [7], where 8-OH-EFV was demonstrated to be a more potent modulator of hepatic cell death than the parent compound; this suggests that **2** may contribute to EFV-mediated hepatotoxicity. More recently, 8-OH-EFV was proved to be at least an order of magnitude more toxic than EFV or 7-OH-EFV in primary rat neurons, which suggests that **2** is a potent neurotoxin [8]. These observations are in accordance with our recent report demonstrating the formation of a quinone derivative (presumably **5**) upon oxidation of 8-OH-EFV (**2**) mediated by a bio-inspired nonheme Fe-complex catalyst [9]. Quinone metabolites are of recognized toxicological significance due not only to their pro-oxidant activity and redox cycling, which yields reactive oxygen species, but also to their reactivity as Michael acceptors, capable of yielding covalent adducts with bionucleophiles [10,11]. Of note, covalent adducts formed upon reaction of quinones with nucleophilic sites of proteins (mainly cysteine residues) have been proposed to be at the onset of both hepatotoxic [12] and neurotoxic [13] events. Taking

Abbreviations: CYP, cytochrome P450; EFV, efavirenz; ESI, electrospray ionization; HIV, human immunodeficiency virus type 1; HPLC-DAD, high performance liquid chromatography with diode array detection; LC–ESI(+)–MS, high performance liquid chromatography with mass spectrometric detection using electrospray ionization in the positive mode; MS/MS, tandem mass spectrometry; NNRTI, non-nucleoside reverse transcriptase inhibitor.

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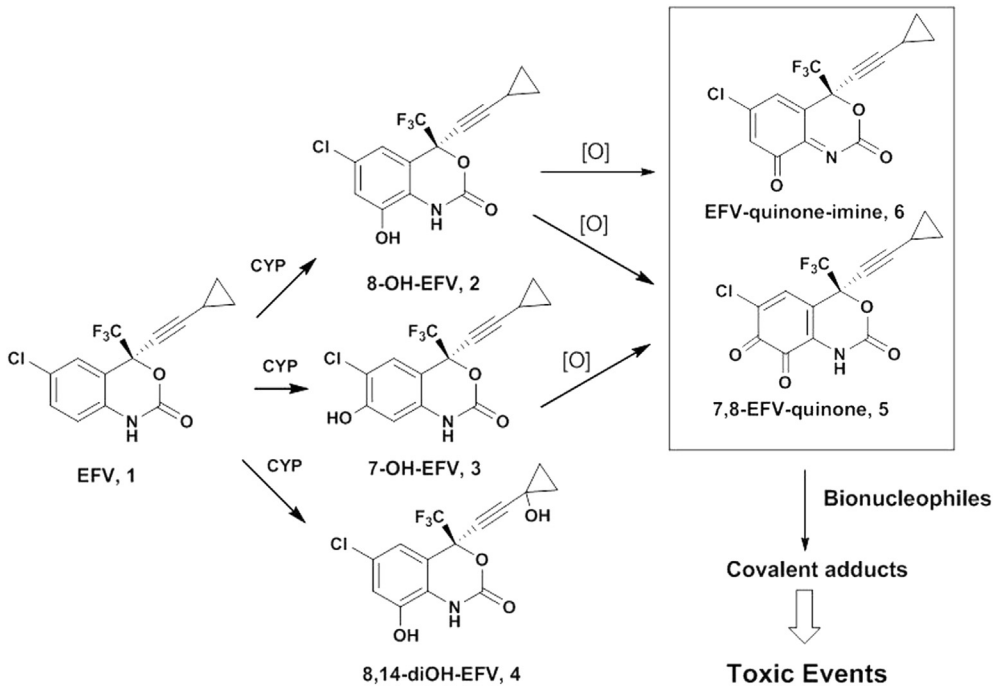


Scheme 1. Efavirenz (**1**) and its phase I metabolites, 8-OH-EFV (**2**), 7-OH-EFV (**3**), and 8,14-diOH-EFV (**4**). Also shown are the putative oxidative bioactivation pathways to the electrophilic quinoid species **5** and **6**, conceivably capable of reacting with bionucleophiles and yield covalent adducts.

into consideration the structural features of the two phenolic EFV metabolites, both **2** and **3** could conceivably generate the ortho-quinone species, 7,8-EFV-quinone (**5**, Scheme 1), upon metabolic oxidation. In contrast, the formation of the potentially toxic quinone–imine electrophile **6** is only possible from 8-OH-EFV. As such, a better understanding of the different behaviours of these two metabolites under oxidative conditions is essential to elucidate if **2** and **3** are both prone to oxidative bioactivation and if different oxidized derivatives of **2** and **3** could play distinct roles in the toxic events associated with EFV administration. Therefore, with the ultimate goal of preparing synthetic standards to investigate the oxidative biotransformation of 7-OH-EFV and 8-OH-EFV *in vivo*, we examined their oxidation *in vitro* using Frémy's salt.

2. Results and discussion

2.1. Oxidation of 7-OH-EFV (3) and 8-OH-EFV (2)

The synthesis of racemic 7-OH-EFV (**3**) was conducted by adaptation of the multi-step procedure reported by Markwalder et al. [14] (*cf.* Supporting information); 8-OH-EFV (2) was prepared by direct oxidation of EFV mediated by a bio-inspired nonheme Fe-complex, as described in Wanke et al. [9]. The oxidation of the two phenolic metabolites was subsequently carried out with the radical potassium nitrosodisulfonate (Frémy's salt), which is frequently employed to obtain quinones from phenolic compounds [15–17]. Additionally, this model oxidant has the advantage of mimicking the processes involved in enzyme-mediated metabolic oxidations [18,19]. The experimental conditions used correspond to those we optimized for the oxidation of a phenolic metabolite of the anti-HIV drug nevirapine [20], consisting on the use of 1.5 eq. of Frémy's salt in a solution of CH_3CN/phosphate buffer (pH 7.4).

Following a 24-h incubation, the quinoline derivative **7** (Scheme 2) was selectively obtained from racemic 7-OH-EFV in 10% yield and was subsequently fully characterized by NMR, MS and X-ray diffraction analysis. In contrast, no evidence for the formation of an isomeric derivative of **7** was obtained upon oxidation of 8-OH-EFV under similar conditions (Fig. 1); instead a more complex mixture was obtained. Thus, the LC–ESI(+)–MS chromatograms from the oxidation of 7-OH-EFV after 5 min and 24 h (Fig. 1B and D) clearly show the selective formation of a product, subsequently identified as the quinoline derivative **7**, corresponding to a signal with a 12.4 min retention time under the elution conditions used (*cf.* Supporting information), and with *m*/*z* 288 $[(^{35}Cl)M + H]^+$ and 290 $[(^{37}Cl)M + H]^+$; the parent compound, 7-OH-EFV, was still present in a significant amount after 24 h. In contrast, the corresponding chromatograms for the oxidation of 8-OH-EFV (Fig. 1A and C) indicate the formation of multiple products. Among these, we were able to identify *m*/*z* values compatible with the protonated molecule of the racemic quinone–imine **6** (*m*/*z* 330 and 332). Further evidence for the formation of this electrophilic metabolite was provided upon LC–ESI–MS/MS analysis of ion *m*/*z* 330. Indeed, MS/MS of this ion yielded a fragment ion at *m*/*z* 266 (Fig. 2), corresponding to the loss of the ethynylcyclopropane moiety from the protonated molecule, along with two other fragment ions at *m*/*z* 302 and 238, corresponding to the loss of CO from the protonated molecule and from the fragment ion at *m*/*z* 266, respectively, which are fully consistent with the assigned structure. This oxidation product was only detected at the beginning of the reaction, which

Scheme 2. Formation of the quinoline derivative **7** upon oxidation of racemic 7-OH-EFV (**3**) with Frémy's salt.

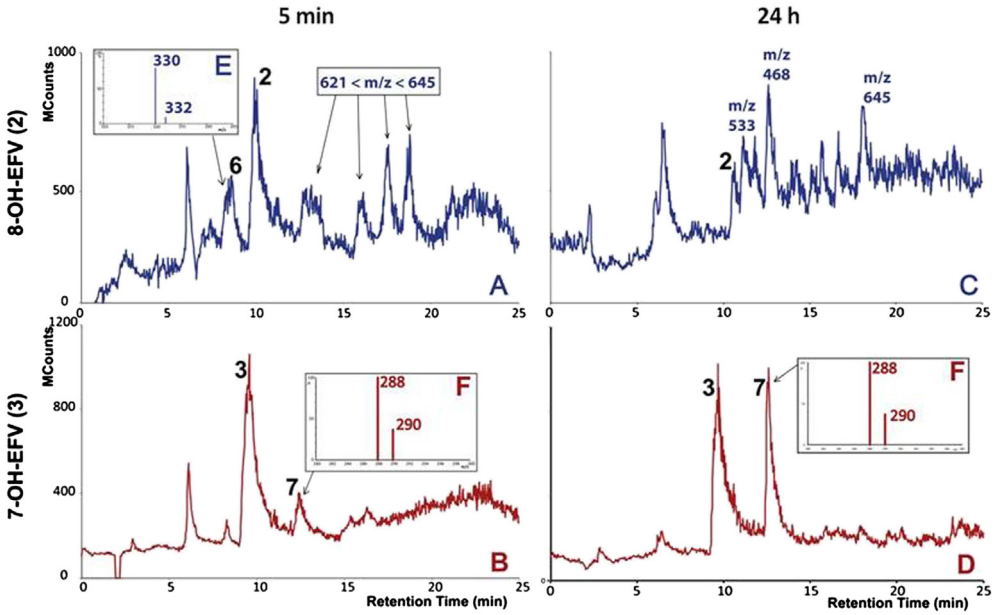


Fig. 1. Comparison of LC–ESI(+)–MS profiles of the reaction mixtures obtained upon oxidation of 8-OH-EFV (**2**) and 7-OH-EF (**3**) with Frémy's salt: A. oxidation of **2** after 5 min; B. oxidation of **3** after 5 min; C. oxidation of **2** after 24 h; D. oxidation of **3** after 24 h; and mass spectra of: E. quinone–imine **6**; and F. quinoline **7**. The elution conditions are outlined in the Supporting information.

demonstrates its transient character. It should be outlined that although we were unable to detect *m*/*z* values compatible with the quinone **5**, its formation under the oxidative conditions used cannot be excluded; in fact, the presumed instability of this electrophilic intermediate may have prevented its detection. Multiple products (with higher *m*/*z* values) were detected both after 5 min and 24 h of 8-OH-EFV oxidation; the LC–ESI(+)–MS chromatographic profiles were considerably different at these two time points, demonstrating the instability of the oxidation products initially formed. Although the relatively high *m*/*z* values corresponding to these species suggest the occurrence of coupling processes, the structural assignment of the oxidation products of 8-OH-EFV was precluded by a number of limitations, including: i) the scarce structural information obtained from tandem mass spectrometry (MS/MS) analysis; ii) the large number (and small amounts) of products, that prevented an efficient chromatographic purification and consequently NMR analysis. One additional distinctive feature between the behaviours of **2** and **3** is their relative stability under the oxidative conditions used. Hence, whereas 8-OH-EFV was only detected in trace amounts after 24 h of incubation (Fig. 1C), approximately 90% of the initial amount of 7-OH-EFV remained unreacted after the same time period (Fig. 1D), in accordance with the isolated yield of the quinoline derivative **7**. Moreover, even with longer incubation times (36 and 48 h), only 7-OH-EFV and the quinoline **7** were detected by HPLC-DAD and LC–ESI(+)–MS analysis of reaction mixtures from the oxidation of 7-OH-EFV (data not shown). Taken together, these observations unequivocally show a remarkable difference in the reactivity of the two phenolic EFV metabolites under oxidative conditions.

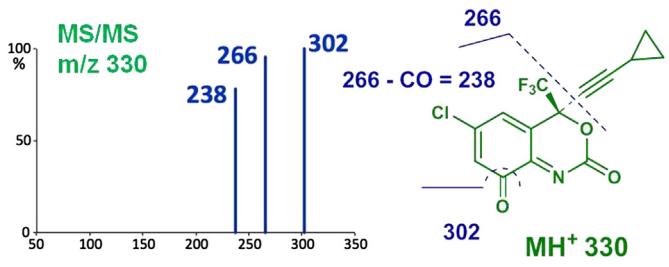


Fig. 2. Tandem mass spectrum (*m*/*z* 330) at retention time 9 min, following LC–ESI(+)–MS/MS analysis of reaction mixture obtained upon 5 min of the oxidation of 8-OH-EFV (**2**) with Frémy's salt.

The considerable stabilities of 7-OH-EFV and its quinoline derivative **7** compared to the relatively short life of 8-OH-EFV under the oxidative conditions used in our work, and the fact that Frémy's salt is often used to mimic the one-electron oxidation steps of enzyme-mediated metabolic oxidations [18,19], suggest that 8-OH-EFV may be more prone than 7-OH-EFV to undergo oxidative bioactivation *in vivo*. Moreover, the MS evidence for the transient formation of an electrophilic quinone–imine (**6**) upon oxidation of 8-OH-EFV, along with the recognized toxicological significance of quinone–imine derivatives [11], suggests that oxidative bioactivation of the major EFV metabolite plays a role in the onset of the toxic events elicited by the parent drug. Indeed, oxidation of 8-OH-EFV to unstable quinoid electrophiles (*i.e.*, **6** and/or **5**) appears as a plausible basis for the distinct toxicity profiles of the two phenolic EFV metabolites in primary rat neurons [8].

On the other hand, the absence of reaction products other than the quinoline **7**, even after prolonged incubation with Frémy's salt, indicates that formation of the electrophilic quinone **5** from 7-OH-EFV is improbable. Additionally, our results suggest that the quinoline **7** is not prone to subsequent aromatic oxidations that might also lead to quinoid structures. Therefore, product **7** (a putative EFV metabolite *in vivo*) is unlikely to have toxicological relevance.

2.2. Spectroscopic characterization of quinoline 7

The ESI(+) mass spectral data for the compound were entirely consistent with structure **7**, with two signals at *m*/*z* 288 and 290, corresponding to the two isotopical protonated molecules [(^{35}Cl) M + H]$^+$ and [(^{37}Cl)M + H]$^+$, respectively. The ^{1}H- and ^{13}C NMR spectra were also compatible with the proposed structure (*cf.* Supporting information); in particular, the high conjugation of the bicyclic aromatic ring allowed the detection of useful ^{1}H–^{19}F and ^{13}C–^{19}F coupling constants (H5–F, J^5_{HF} = 1.8 Hz; C3–F,

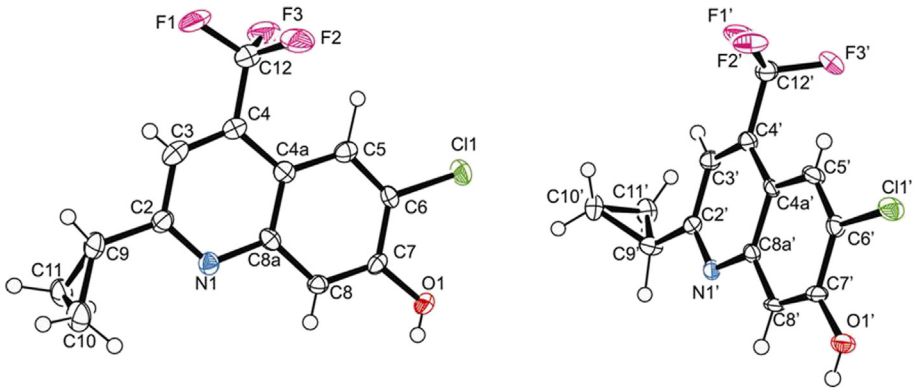


Fig. 3. ORTEP plot representation drawn with 50% probability, showing the atomic labelling scheme for compound **7** in the two distinct asymmetric units identified in the cell.

$J^3_{CF} = 5.0$ Hz). However, the lack of informative $^1H-^{13}C$ three-bond correlations in the HMBC spectrum precluded a definitive structural assignment based exclusively on NMR experiments. This could only be possible by X-ray diffraction, which showed unambiguously that the product from oxidation of 7-OH-EFV was 6-chloro-2-cyclopropyl-4-(trifluoromethyl)quinolin-7-ol (**7**, Fig. 3).

Bond lengths (Å) and angles (°) in the structure of **7** were in accordance with the expected values for quinoline compounds [21]: the internal angles of the condensed rings were in the range of 115.9(4)–122.7(5)° and the bicyclic cores were nearly planar (angles between the <C4a–C5–C6–C7–C8–C8a> and <C2–C3–C4–C4a–C8a–N1> planes and between the <C4a′–C5′–C6′–C7′–C8′–C8a″> and <C2′–C3′–C4′–C4a′–C8′–N1′> planes were 3.27° and 1.93°, respectively). Crystal packing revealed two intermolecular N···H–O H-bonds for each asymmetric unit, namely between N1 of one unit and the H1–O1 of the adjacent molecule that links to the previous and the subsequent element, forming a 3D-chain (see Fig. S1 in the Supporting information). The latter appeared to be reinforced in the crystal packing by two π-stacking interactions for both asymmetric units, specifically between the centroid of each unit (*i.e.*, C4a–C5–C6–C7–C8–C8a and C2′–C3′–C4′–C4a′–C8a′–N1′) and the analogues of the adjacent unit (*i.e.*, C2–C3–C4–C4a–C8a–N1 and C4a′–C5′–C6′–C7′–C8′–C8a′, correspondingly).

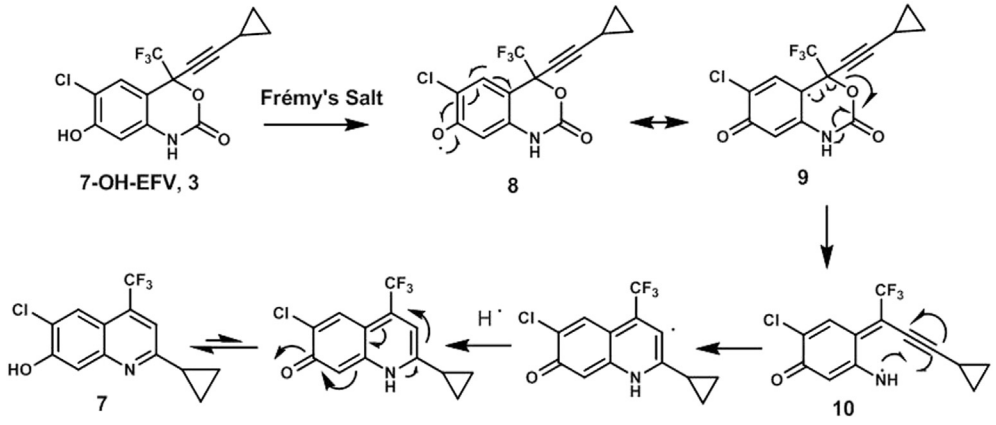
2.3. Proposed mechanism for the formation of quinoline 7

The identification of trace amounts of **7** upon HPLC-DAD analysis of a solution of 7-OH-EFV (**3**) in CH_3CN/50 mM phosphate buffer (pH 7.4), in the absence of oxidant, suggests that this phenolic derivative can (to some extent) be oxidized by molecular oxygen (not shown). This observation supports the hypothesis that **7** was formed by a radical mechanism [22]. As such, the ring opening mechanism is proposed to be induced by the initial formation of an unstable phenoxyl radical **8**, generated upon oxidation of 7-OH-EFV (**3**) by Frémy's salt radical (Scheme 3).

This phenoxyl radical is a resonance hybrid with an activated *para*-position (contributor **9**), which subsequently undergoes homolytic bond cleavage of the carbamate moiety, assisted by the release of CO_2. The resulting intermediate **10** is proposed to undergo subsequent cyclization, followed by aromatization to yield the quinoline core. The formation of quinoline derivatives by analogues of the intermediate **9** [14,23–25] supports this hypothesis.

3. Conclusions

The quinoline derivative **7** was selectively obtained upon oxidation of the phenolic metabolite of EFV, 7-OH-EFV (**3**), with Frémy's salt. Our data indicate that this oxidative pathway of **3** is favoured over the conceivable formation of the potentially toxic quinone **5**, that was previously identified by our group in the oxidation of 8-OH-EFV (**2**) with a bioinspired non-heme Fe(II) catalyst. One additional potentially toxic metabolite, the quinone–imine **6**, was tentatively identified upon oxidation of 8-OH-EFV with Frémy's salt. These contrasting behaviours under oxidative conditions may explain the distinct toxicities of the two phenolic EFV metabolites observed in primary rat neuron cultures [8]. Indeed, when formed metabolically, both quinones and quinone–imines are electrophilic intermediates of unquestionable toxicological significance, due not only to their pro-oxidant activity and redox cycling ability, which contribute to generate reactive oxygen



Scheme 3. Proposed mechanism for the formation of the quinoline derivative **7**, upon oxidation of 7-OH-EFV (**3**) with Frémy's salt.

species, but also to their reactivity toward bionucleophiles, as Michael acceptors [10,11]. The plausibility of this bioactivation pathway and its pivotal role in the onset of toxic events induced by several toxicants is illustrated by a few selected examples: (1) the CYP-mediated oxidation of paracetamol to the electrophilic metabolite *N*-acetyl-*para*-benzoquinone-imine is amply recognized to be linked with the hepatotoxic events induced by this analgesic drug [26]; (2) the enzyme tyrosinase mediates the oxidation of L-DOPA to a quinone metabolite that is proposed to be linked with the neurotoxicity induced by this endogenous metabolite *via* formation of covalent adducts with proteins [13]; (3) the conversion of diclofenac into quinone–imine and quinone–methide metabolites upon CYP450 bioactivation is considered an important factor in the pathogenesis of the idiosyncratic hepatotoxicity induced by this nonsteroidal anti-inflammatory drug [27]; and (4) myeloperoxidases are responsible for the ultimate activation of some phenolic metabolites of benzene into quinones that account in part for the carcinogenicity of benzene and the numerous cases of benzene-induced leukemia [28].

Taking these data together, along with the fact that 8-OH-EFV is the major EFV metabolite in humans, the bioactivation pathway to the electrophilic quinone–imine **6** (and/or the quinone **5**), is likely to occur *in vivo* and is expected to have a key role in the onset of the EFV-induced toxic events.

The CYP450-mediated formation of phenoxide radicals is a frequent event during the bioactivation of phenolic metabolites [29]; this fact sets the *in vivo* formation of quinoline **7** as highly probable. However, based on the stability of the quinoline **7** under the oxidative conditions used in the current study, no toxicological significance is expected to arise from oxidative bioactivation of this derivative. Nonetheless, the availability of this fully characterized standard, which will conceivably be formed *in vivo*, is important to the establishment of accurate pharmokinetic parameters of the first line antiretroviral drug EFV. This new EFV derivative (and putative metabolite) is now accessible for further molecular toxicology studies aimed at clarifying the distinct relevances of the two phenolic EFV metabolites and their oxidation products to the toxic events associated with the parent drug.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.12.022.

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