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Mechanisms of trovafloxacin hepatotoxicity: Studies of a model cyclopropylamine-containing system

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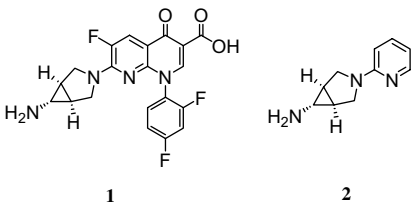
Abstract—The mechanism for the hepatotoxicity of trovafloxacin remains unresolved. Trovafloxacin contains a cyclopropylamine moiety which has a potential to be oxidized to reactive intermediate(s) although other putative elements may exist. In this study, a drug model of trovafloxacin containing the cyclopropylamine substructure was synthesized. Chemical oxidation of the drug model by $K_3Fe(CN)_6$ and NaClO revealed that both oxidants oxidize this drug model to a reactive α,β-unsaturated aldehyde, **11**. The structure of **11** was fully elucidated by LC/MS/MS and NMR analysis. These results suggested that P450s with heme-iron center and myeloperoxidase generating hypochlorous acid in the presence of chloride ion are capable of bioactivating the cyclopropylamine moiety of trovafloxacin. This deleterious metabolism may lead to eventual hepatotoxicity.

Trovafloxacin (Trovan™) **1** is a naphthyridone agent of the fluoroquinolone antibiotics. Major advantages of **1** over traditional agents such as ciprofloxacin and later fluoroquinolone sparfloxacin lie in its broader spectrum and superior pharmacokinetic profiles.[1] Since **1**'s marketing in 1998, rare but severe hepatotoxicities have been reported, which ultimately led to Trovan's withdrawal in some countries and a black-box warning in United States since 1999.[2] Clinical reports characterized the hepatotoxicity of trovafloxacin as an idiosyncratic drug reaction since it was unpredictable and possibly immunologically based.[3]

The etiology of idiosyncratic hepatotoxicity involves multiple factors. It is well recognized that metabolic activation of some drugs may lead to the formation of a reactive species capable of covalent binding to proteins, ultimately culminating in a toxic response.[4] Structurally, trovafloxacin possesses two elements that have the potential to generate reactive intermediate(s): a cyclopropylamine moiety and a difluoroanilino system. The cyclopropylamine moiety is the most suspect substructure to induce hepatotoxicity of trovafloxacin. Previous studies showed that P450s,[5–8] monoamine oxidases (MAOs),[9–12] and horseradish peroxidase (HRP)[13] can all oxidize cyclopropylamine to a carbon-centered radical, which can be subsequently oxidized to a reactive α,β-unsaturated aldehyde. Although the difluoroanilino functional group was suggested to generate reactive metabolites,[14] there has been no corroborating experimental evidence. To exclude interferences from other possible oxidizable positions, especially the difluoroanilino moiety, a drug model of trovafloxacin **2** (Fig. 1) containing the cyclopropylamine substructure was synthesized. Chemical oxidations, which mimic the oxidation modes of different enzymes, were then performed to help propose responsible enzymes that can oxidize **2** and also to investigate reactive intermediate(s) that can be generated from the respective oxidation.



1 **2**

Figure 1. Structures of trovafloxacin (Trovan™) **1** and the drug model of trovafloxacin **2**. The bold lines highlight the similarities between trovafloxacin and the drug model.

Keywords: Trovafloxacin; Idiosyncratic drug reaction; Cyclopropylamine; $K_3Fe(CN)_6$; NaClO; P450s; MPO.
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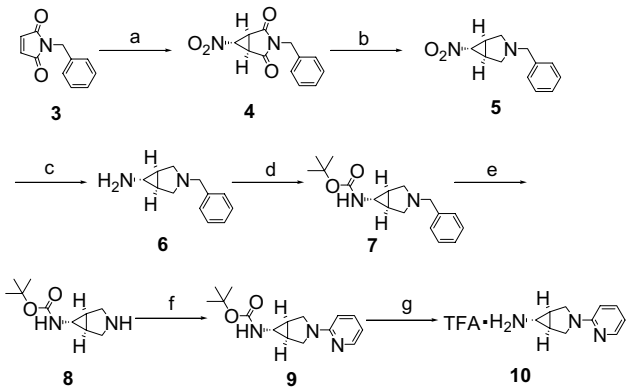


Scheme 1. Reagents and conditions: (a) CH_2BrNO_2, K_2CO_3, ACN; (b) BH_3 * THF, THF, reflux; (c) Zn, 1 N HCl, *i*-PrOH; (d) Boc_2O, TEA, DCM; (e) H_2, 20% $Pd(OH)_2$/C, MeOH; (f) 2-fluoropyridine, TEA, 90 °C; (g) 50% TFA/DCM.

$K_3Fe(CN)_6$ was chosen as a single electron transfer (SET) oxidant, mimicking heme-iron centered enzymes such as P450s. NaClO was applied to mimic myeloperoxidase (MPO), which can generate hypochlorous acid in the presence of chloride ion. Our interest in the oxidation of **2** by MPO arose from the fact that neutrophils, which release MPO, have been associated with idiosyncratic drug reaction of trovafloxacin in a LPS-trovafloxacin idiosyncrasy animal model.[15]

Monoamine oxidases (MAOs) should also be considered for the bioactivation of the drug model, especially considering that MAOs oxidize a variety of primary cyclopropylamines to reactive species.[9–12] Some flavin models, such as 3-methyllumiflavin[16] and 3-methyl-5-ethyllumiflavinium perchlorate,[17] have been applied to mimic the oxidation of MAOs. However, these model systems failed to fully mimic MAO activity. MAOs cat-

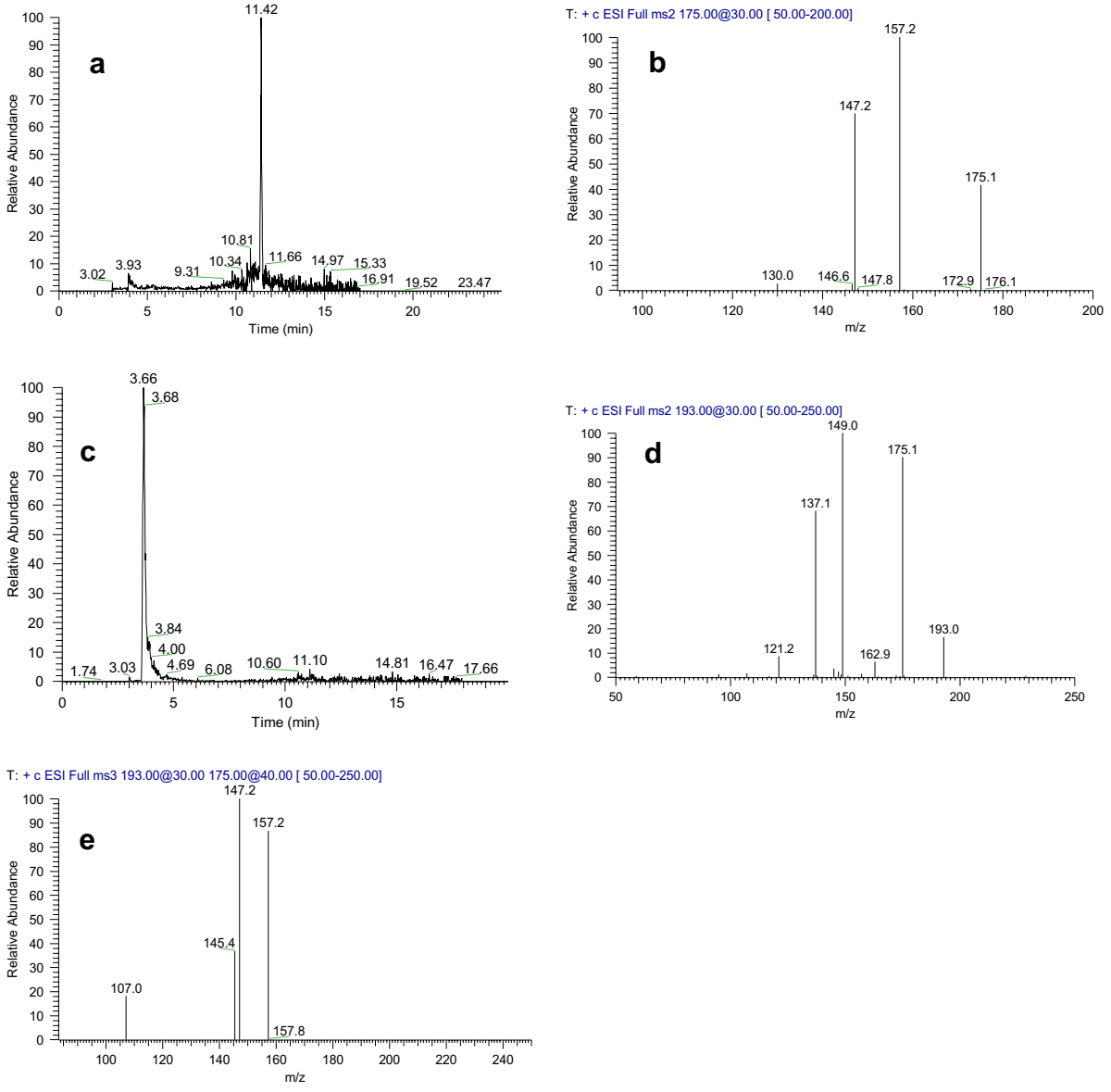


Figure 2. LC chromatogram and mass spectrum of oxidized products by $K_3Fe(CN)_6$with MH^+ at 175 and 193. (a) LC chromatogram of 175 from oxidation by $K_3Fe(CN)_6$ for 5 h. (b) MS/MS spectrum of 175. (c) LC chromatogram of 193 from oxidation by $K_3Fe(CN)_6$ for 10 h. (d) MS/MS spectrum of 193. (e) MS^3 spectrum of 175 from MS^2 of 193.

alyze the oxidation of primary, secondary, and tertiary amines. The models oxidized primary amines, but not tertiary amine substrates.[16,17] Considering the fact that there is still some uncertainty about the mechanism of oxidation by MAOs, the known mimics are not suitable as oxidation models for mimicking the bioactivation of MAOs.

The synthesis of **2** is illustrated in Scheme 1. The exo-nitrocyclopropane derivative **4** was synthesized by reaction of *N*-benzylmaleimide **3** with bromonitromethane in the presence of a base. Earlier trials of the same transformation with a variety of bases gave yields lower than 15%.[18] A modified method using 1,2-dimethyl-1,4,5,6-tetrahydropyrimidine (DMTHP) improved the yield to 30–35%. However, as reported by the authors, a drawback of the reaction is the tar formation due to the undesired side reaction of the base with both the *N*-benzylmaleimide and the bromonitromethane.[19] Ballini et al.[20] increased the yield of this reaction to 70% by using potassium carbonate as base and acetonitrile as solvent. Addition of 1.5 equivalent of bromonitromethane in portions over 20 h was claimed to be essential for a good yield. In our experiment, 1.2 equivalent of bromonitromethane was added dropwise over 5 h, and a 67% yield was obtained. Imide carbonyl groups of **4** were then reduced by borane·THF complex to give **5**. Cyclopropyl nitro group of **5** was subsequently reduced by Zn and 1 N HCl (aq) with *i*-PrOH as co-solvent to produce the primary cyclopropylamine **6**.[21] The primary amine was protected by Boc to give **7**, and **7** was then debenzylated to **8** by hydrogenation, using $Pd(OH)_2/C$ as catalyst. Selectively protected secondary amine **8** was then coupled with 2-fluoropyridine to generate **9**. Boc deprotection of **9** provided the drug model of trovafloxacin in TFA salt form **10**. The stereochemistry of cyclopropylamine has been reported to favor the exo-form to a very significant extent and the exo/endo ratio is approximately 98:2.[22]

Chemical oxidation of the drug model by $K_3Fe(CN)_6$ was performed as follows. Ten milligrams of compound **9** was deprotected prior to use by 50% TFA/CH_2Cl_2 to give 3.64×10^{-5} mol drug model in TFA salt form. The dried residual oil was dissolved in 300 μl MeOH and 400 μl H_2O. The pH was adjusted to 9 by 150 μl of 1 M NaOH and 24 mg $K_3Fe(CN)_6$ (2 equiv) was added. After 5 h, a new peak from LC/MS analysis[23] was observed with $t_R = 11.42$ min (Fig. 2a) and MH^+ at *m*/*z* 175. MS/MS analysis showed that 175 gave rise to typical aldehyde fragments at 157 (loss of H_2O) and 147 (loss of CO) (Fig. 2b). After the oxidation was run for 10 h, another product with $t_R = 3.68$ min (Fig. 2c) and MH^+ at *m*/*z* 193 appeared, which was not observed at 5 h. Fragmentation of 193 generated product peaks at 175, 163, 149, 137, and 121 (Fig. 2d). Further fragmentation of product peak 175 gave peaks at 157, 147, 145, and 107 (Fig. 2e). The proposed structures for 175 and 193 are shown in Figure 3 and the principal mass spectral fragments were also assigned. Compound **12** can be formed by adding water to the α,β-unsaturated aldehyde **11** via Michael addition. To confirm the structure of the reactive α,β-unsaturated aldehyde **11**, $K_3Fe(CN)_6$ oxidation was scaled up, providing ample amounts of pure **11**, which were isolated for ^1H and ^{13}C NMR analysis. The NMR assignment of **11**, as summarized in Table 1, confirmed the structure proposed based on MS/MS analysis. These results demonstrated that $K_3Fe(CN)_6$ can oxidize the drug model **2** to a reactive α,β-unsaturated aldehyde **11**, suggesting that enzymes with heme-iron center, such as P450s, may bioactivate the cyclopropylamine moiety of trovafloxacin and possibly be responsible for the toxicity.

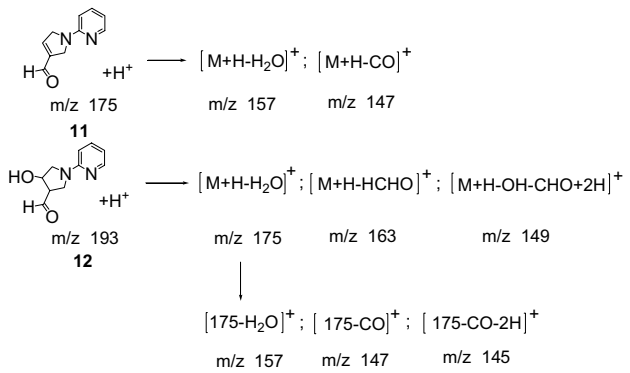
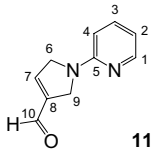


Figure 3. Proposed principal mass spectral fragments of 175 and 193.

Chemical oxidation of drug model by NaClO was also performed. To date, there has been no reported oxidation of cyclopropylamines by MPO, although large numbers of normal aliphatic and aromatic amines are good substrates of MPO. In that scenario, identifying the possible reactive intermediate(s) from oxidation of cyclopropylamine by MPO is crucial in understanding the influence of neutrophils on the toxicity of trovafloxacin in the animal model.[14] Chemical oxidation by NaClO was carried out in the same way as $K_3Fe(CN)_6$ oxidation except 430 μl NaClO (aq) containing 0.5% chlorine (1 M equiv) was added instead of $K_3Fe(CN)_6$.

Table 1. ^1H NMR and ^{13}C NMR ($CDCl_3$) assignments for reactive α,β-unsaturated aldehyde **11**



11

Position	δ_C[a]	δ_H[a] (multiplicities,[b] *J* (Hz))
1	148.5	8.12 (d, 5.1)
2	112.6	6.56 (dd, 5.1, 7.2)
3	137.7	7.44 (m)
4	106.4	6.34 (d, 8.7)
5		
6	54.8	4.54 (m)
7	145.1	6.96 (m)
8	142.8	
9	51.0	4.34 (m)
10	187.7	9.77 (s)

[a] Chemical shifts in ppm.
[b] Notations: dd, doublet of doublets; m, multiplet; d, doublet; s, singlet.

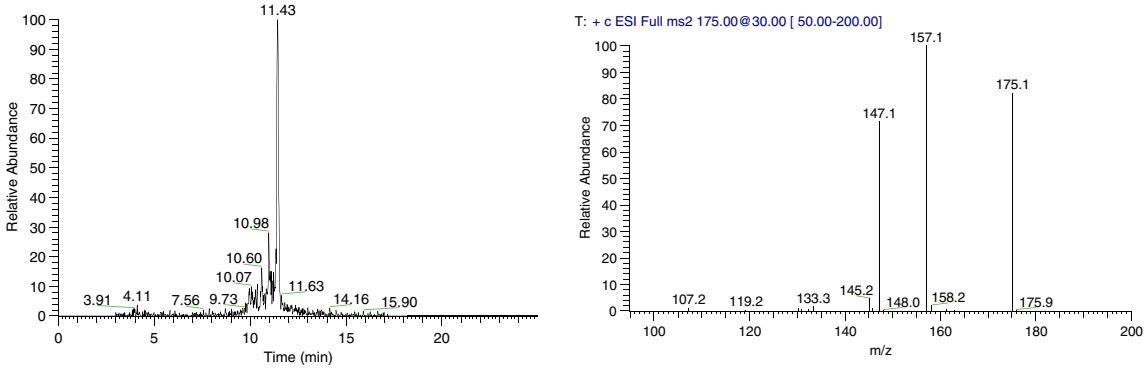


Figure 4. Left panel: LC chromatogram with mass filter *m/z* 175 showing peak (t_R = 11.43 min) associated with oxidized product **11** by NaClO, which has same retention time as Figure 2a. Right panel: MS/MS spectrum of oxidized product **11** by NaClO, which yields the same fragment ions as Figure 2b.

The LC/MS analysis[23] showed that an oxidized product with MH^+ at 175 was also generated by NaClO oxidation. The product showed the same retention time and fragment ions (Fig. 4) as **11** from $K_3Fe(CN)_6$ oxidation, which means both NaClO and $K_3Fe(CN)_6$ can oxidize the drug model to the reactive α,β-unsaturated aldehyde **11**. This suggests that MPO is also possibly responsible for the hepatotoxicity of trovafloxacin.

The mechanism of idiosyncratic hepatotoxicity of trovafloxacin has intrigued both academia and industry for more than 8 years. There are several oxidizable positions that can be bioactivated and lead to the toxicity of trovafloxacin. To simplify the problem, a drug model of trovafloxacin containing the cyclopropylamine substructure was synthesized and the chemical oxidations by $K_3Fe(CN)_6$ and NaClO were performed. The results presented here showed that both $K_3Fe(CN)_6$ and NaClO can oxidize the drug model to a reactive α,β-unsaturated aldehyde **11**, implicating that heme-iron centered enzymes such as P450s and MPO may possibly be responsible enzymes for the hepatotoxicity of trovafloxacin. Chemical oxidation provides an economic and efficient way of probing the possible reactions of cyclopropylamine and is important for helping to choose the expensive biological enzymes for in vitro metabolism research. Considering the limited amount of metabolite generated from enzyme metabolism, full structure elucidation of the reactive metabolite or its conjugate by NMR is typically challenging and expensive as a result of needing to isolate the product from enzyme metabolism mixtures. In addition, the total synthesis of the α,β-unsaturated aldehyde **11** is also not readily available. Chemical oxidation proved to be a good way to provide a standard compound for structure elucidation of in vitro enzyme metabolites. Under the guidance of chemical oxidation results, especially with the useful information provided by the chemical oxidized product—α,β-unsaturated aldehyde **11**, our subsequent in vitro enzyme metabolism studies of the drug model have revealed that one P450 enzyme and also MPO are able to oxidize the drug model to the reactive α,β-unsaturated aldehyde **11** and may be the responsible enzymes for the toxicity. The detailed results for in vitro enzymatic metabolism of the drug model and also trovafloxacin will be reported separately.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.10.070.

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