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Assessment of the genotoxicity of quinolone and fluoroquinolones contaminated soil with the *Vicia faba* micronucleus test

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ABSTRACT

The genotoxicity of quinolone and fluroquinolones was assessed using the micronucleus (MN) test on *Vicia faba* roots by direct contact exposure to a solid matrix. Plants were exposed to quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin and enrofloxacin) alone or mixed with artificially contaminated soils. Four different concentrations of each of these antibiotics were tested (0.01, 0.1, 1 and 10 mg/Kg) for nalidixic acid and (0.005, 0.05, 0.5 and 5 mg/Kg) for ciprofloxacin and enrofloxacin. These antibiotics were also used in mixture. Exposure of *Vicia faba* plants to each antibiotic at the highest two concentrations showed significant MN induction. The lowest two concentrations had no significant genotoxic effect. The mixture of the three compounds induced a significant MN induction whatever the mixture tested, from 0.02 to 20 mg/Kg. The results indicated that a similar genotoxic effect was obtained with the mixture at 0.2 mg/Kg in comparison with each molecule alone at 5–10 mg/Kg. Data revealed a clear synergism of these molecules on *Vicia faba* genotoxicity.

1. Introduction

In recent years, public and scientific concern about the relevance of trace amounts of pharmaceuticals that occur in the environment has been continuously increasing (Pico and Andreu, 2007). Numerous pharmaceuticals have been detected in waste and natural water resources, sediments, soil and aquatic biota. While the pharmaceuticals are found at relatively low concentrations (Terns, 1998; Korpin et al., 2002), elevated concentrations of antibiotics (several mg/Kg levels) have been reported in manure (Hamscher et al., 2002), sewage sludge and sewage-treated soil (Golet et al., 2003). Indeed after administration to humans, fifty to ninety percent of these pharmaceuticals or their primary metabolites are rapidly excreted into wastewater. In previous works (Golet et al., 2002, 2003), Fluoroquinolones (FQs) in raw sludge, wastewater effluents and river water samples were analyzed in Switzerland. They found out that a large proportion of FQs entering wastewater plants (89–92% of the FQs mass flow) was removed during the treatment processes. They also suggested that sewage sludge was the main source and reservoir of FQs residues (Golet et al., 2003). Sewage sludge is dispersed on the fields and the antibiotics may contaminate the soil and eventually the ground water (Hamscher et al., 2005).

Many drugs used in hospitals (antibiotics, cytostatic drugs) are designed to exhibit DNA damage toward bacteria or eukaryotic cells, raising concern about the human and ecological hazard of hospital wastewater (Giuliani et al., 1996). Ciprofloxacin was found in concentration ranging from 0.7 to 124.5 μg/L in hospital effluents and was assumed to be the main source of genotoxic effects measured with the UmuC test in these effluents (Hartmann et al., 1999). Fluoroquinolones have been reported to induce unscheduled DNA synthesis, DNA strand breakage, chromosome damage and micronuclei formation (Mc Queen et al., 1991; Holden et al., 1989; Bredberg et al., 1991; Ciaravinco et al., 1993; Curry et al., 1996; Gibson et al., 1998). In addition, FQs exert other toxic and genotoxic effects on animal and human cells. For example, exposure of Chinese hamster ovary cells or Syrian hamster embryo cells to nalidixic acid (NA) and ciprofloxacin (CIP) induced cytotoxic effects and micronucleus induction (Gibson et al., 1998). In vitro studies with human lymphocytes exposed to enrofloxacin (ENR) and ciprofloxacin revealed an

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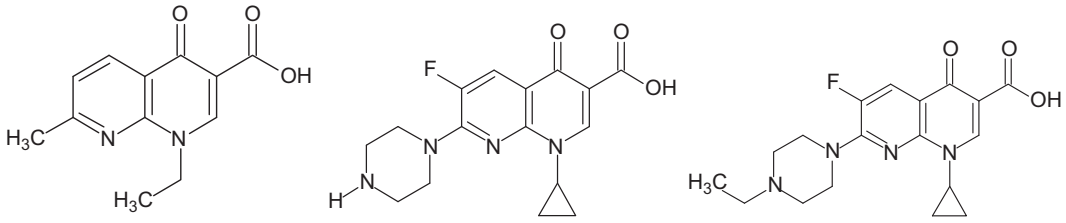

Fig. 1. Chemical structures and molecular weights of the antibiotics studied.

increase in the chromosomal aberrations, detected as chromatid and chromosome breaks and gaps (Gorla et al., 1999). FQs have been reported to cause neonatal alterations in articulation cartilages, bone growth and tendons, both in humans and animals (Patterson, 1991; Gough et al., 1992; Hildebrand et al., 1993; Forster et al., 1996; Simonin et al., 1999; Stahlmann, 2003; Lemus et al., 2009).

The *Vicia faba* micronucleus test has been shown to be sensitive in evaluating chromosomal aberrations and assessing genotoxicity from both organic and inorganic soil contaminants (Cotelle et al., 1999), sediment (Chen and White, 2004), organic material such as sewage sludge or composts (De Simone et al., 2000) and water (Miao et al., 1999; Monarca et al., 2004). No study has been conducted to test the genotoxic potential of quinolones and fluoroquinolones on *Vicia faba* plant roots. Because these molecules are significantly present in septic tank effluents pumped (STEP) wastewater and sludge, and because some countries use these products in plant cultivation, these antibiotics and more particularly quinolones and fluoroquinolones became a real environmental concern. In this work, the genotoxicity of quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin and enrofloxacin) (Fig. 1), alone or in mixture, were evaluated using the *Vicia faba* micronucleus test by direct contact with artificially contaminated soils.

2. Materials and methods

2.1. Chemicals and reagents

Ciprofloxacin (CIP), enrofloxacin (ENR), nalidixic acid (NA) and maleic hydrazide (MH) were purchased from Sigma-Aldrich. The purity of the chemicals used in this study was $>95\%$ for all of the antibiotics. All the other chemicals used were of analytical grade. Stock solutions of ciprofloxacin, enrofloxacin, nalidixic acid and mixture were prepared in deionised water.

2.2. Antibiotic analysis

Antibiotic concentrations were checked by LC/UV/MS/MS. Chromatographic analyses were performed on a Thermofinnigan Surveyor® HPLC system with diode array detector (DAD) and a LCQ Deca XP Max® ion trap mass spectrometer (Thermo Electron Corporation, Waltham, Mass, USA). Separation was carried out with a Luna C18 column (100×2.0 mm; 3 μm, Phenomenex, Torrance, CA, USA) at 40 °C. A gradient elution was used at a flow rate of 200 μL/min with a mobile phase of acetonitrile 0.1% formic acid (A) and water 0.1% formic acid (B) in the following conditions: 0–1 min, 90% A; 1–8 min, 40% A; 8–10 min, 40% A; 10–11 min, 90% A and 11–15 min, 90% A. The DAD wavelengths were set at 254 nm and 295 nm and molecules were ionized with an electrospray ionization source in positive mode (ESI+). The spray needle was set at a potential of 5 kV. Capillary voltage and temperature were 10 V and 350 °C, respectively. Sheath gas and auxiliary gas flow rate of nitrogen were set at 45 and 10 (arbitrary units), respectively. Helium was used in the trap as damping and collision gas. Collision energies (E_{coll}) were optimized for each antibiotic (Table 2A). CIP, ENR and NA were assayed by HPLC with UV detection and mass spectrometry (MS) using the selection reaction monitoring mode (SRM). The detection parameters are reported in Table 2A.

In mass spectrometry, the parent ion of CIP, ENR and NA was the protonated molecular ions $[M+H]^+$ and their fragment ions were $[M+H\text{-}18]^+$ and/or $[M+H\text{-}44]^+$ corresponding to the loss of H_2O and CO_2, respectively, in accordance with the fragmentations reported in the literature (Yang et al., 2008). Antibiotic preparation solution and mixture were directly quantified before soil contamination with either UV detection for the higher nominal concentrations of 1 or 2 and 10 or 20 mg/L (calibration range: 0.5–50 mg/L) or mass spectrometry for the lower concentrations of 0.1 or 0.2 and 1 or 2 mg/L (calibration range: 0.1–10 mg/L).

Table 1
Key properties of the LUFA standard soil.

Organic carbon (%)	2.33
pH (soil:water, 1:2.5)	5.7
Cation exchange capacity (mval/100 g)	11
Particle size (mm) distribution according to USDA (%)	
<0.002	7.9
0.002–0.05	14.2
0.05–2.0	77.9
Soil type	Loamy sand

Table 2A
Chromatographic, UV detection and MRM parameters used for quinolone quantification in preparation solution and in mixture.

	UV detection		MS detection		
	tr ± SD ($n=6$) (min)	λ_{abs} (nm)	tr ± SD ($n=6$) (min)	MRM transitions	Ecoll (%)
Nalidixic acid	9.63 ± 0.008	254	9.87 ± 0.014	233 < 233 233 < 215	38
Enrofloxacin	4.89 ± 0.030	295	5.12 ± 0.034	360 < 342 360 < 316	36
Ciprofloxacin	3.37 ± 0.031	295	3.60 ± 0.029	332 < 314 332 < 288	38

2.3. Vicia faba micronucleus test

LUFA standard soil was used in all direct contact experiments (Song et al., 2007). Some key characteristics of the LUFA standard soil are presented in Table 1. The *Vicia faba* seeds were prepared according to Ma et al. (1995), El Hajjouji et al. (2007) and Marcato-Romain et al. (2009). Dry *Vicia faba* seeds were soaked for 24 h in deionised water, the seed coats were removed and the seeds left to germinate between two layers of moist cotton. After 5 day, the primary roots, about 2–3 cm in length, were selected for the MN assay and their tips were cut off to promote the growth of the secondary roots. For each experiment, five plants were used as five independent replicates per treatment.

After the germination period, the direct contact method developed by Marcato-Romain et al. (2009) was used by placing germinated roots in the LUFA standard soil for the assessment of the effect of the three antibiotics. Four different concentration levels of each of these molecules were tested (0.01, 0.1, 1 and 10 mg/Kg) for NA and (0.005, 0.05, 0.5 and 5 mg/Kg) for CIP and ENR. A two-fold higher concentration range was used for NA because its potency against susceptible bacteria is at least two-fold lower than that of CIP and ENR. For each concentration, five replicates were processed. These antibiotics were also applied in mixture. In these cases, the mixture 0.02 mg/Kg corresponds to the mixture of NA at 0.01 mg/Kg, CIP and ENR at 0.005 mg/Kg. The same mixtures were realized with final concentrations of 0.2, 2 and 20 mg/Kg of each of these compounds.