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Initial hazard screening for genotoxicity of photo-transformation products of ciprofloxacin by applying a combination of experimental and *in-silico* testing☆

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ABSTRACT

Ciprofloxacin (CIP) is a broad-spectrum antibiotic found within μg/L concentration range in the aquatic environment. It is a known contributor of *umuC* induction in hospital wastewater samples. CIP can undergo photolysis to result in many transformation products (TPs) of mostly unknown toxicity. The aims of this study were to determine the genotoxicity of the UV mixtures and to understand the possible genotoxic role of the stable TPs. As such, CIP and its UV-irradiated mixtures were investigated in a battery of genotoxicity and cytotoxicity *in vitro* assays. The combination index (CI) analysis of residual CIP in the irradiated mixtures was performed for the umu assay. Further, Quantitative Structure–Activity Relationships (QSARs) predicted selected genotoxicity endpoints of the identified TPs. CIP achieved primary elimination after 128 min of irradiation but was not completely mineralized. Nine photo-TPs were identified. The irradiated mixtures were neither mutagenic in the Ames test nor genotoxic in the *in vitro* micronucleus (MN) test. Like CIP, the irradiated mixtures were *umuC* inducing. The CI analysis revealed that the irradiated mixtures and the corresponding CIP concentration in the mixtures shared similar *umuC* potentials. QSAR predictions suggested that the TPs may be capable of inducing chromosome aberration, MN *in vivo*, bacterial mutation and mammalian mutation. However, the experimental testing for a few genotoxic endpoints did not show significant genotoxic activity for the TPs present as a component of the whole mixture analysis and therefore, further genotoxic endpoints may need to be investigated to fully confirm this.

1. Introduction

The risk to human and ecosystem health from the presence of pharmaceuticals as micropollutants has remained a debatable issue in the scientific community (Richardson and Ternes, 2011). This concern is partially due to the sparsity in chronic toxicity data from environmental exposures (Taylor and Senac, 2014). Of particular interest are the antineoplastics and antibiotics drugs since by design they are intended to be toxic to cells (Taylor and Senac, 2014; Toolaram et al., 2014; Bergheim et al., 2015). In this regard, monitoring of not only the inherent cytotoxicity but also genotoxicity nature would be paramount to a risk assessment scheme as many of these drugs can directly or indirectly interact with deoxyribonucleic acid (DNA) (Toolaram et al., 2014). Amongst the antibiotic pharmaceuticals is ciprofloxacin (CIP), a broad-spectrum second-generation fluoroquinolone (FQ) that was identified as the main source of *umuC* genotoxicity in the hospital wastewater investigated by Hartmann et al. (1998). Over the years, it has been detected frequently within the μg/L concentration range in the aquatic environment (Hartmann et al., 1998; Martins et al., 2008).

The mode of action (MOA) of CIP involves the binding of the quinolone moiety to the bacterial DNA gyrase which leads to the stabilization of the cleavable complex, preventing the enzyme turnover and thereby inhibiting the resealing of DNA strand breaks (Clerch et al., 1992; Albertini et al., 1995). The formation of intra- and inter-strand adducts could arrest DNA replication, inducing the

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SOS system and producing lesions that would prompt excision repair (Clerch et al., 1996). Quinolones binds differently to eukaryotic topoisomerase II mainly because of the difference in structural DNA and therefore the genotoxic potential is lower in eukaryotic organisms than prokaryotic organisms (Albertini et al., 1995; Clerch et al., 1996). The MOA of CIP in eukaryotic organisms is believed to be the same as in bacteria resulting in DNA strand breaks that if not repaired can lead to clastogenicity and/or cytotoxicity (Lynch et al., 2003). DNA damage using *in vitro* assays includes micronucleus (MN) formation, chromosome aberration (CA), unscheduled DNA synthesis, induction of HPRT mutation cells and thymidine kinase (TK) mutation (Bredberg et al., 1989; Albertini et al., 1995; Chételat et al., 1996; Curry et al., 1996; Gibson et al., 1998; Lynch et al., 2003; Garcia-Käufer et al., 2012).

Environmental monitoring of FQs including CIP showed that their lower concentrations in sewage effluent compared to raw sewage could be attributed to the sorption process in the wastewater treatment plant (WWTP) (Giger et al., 2003). Therefore, an improvement in FQ removal from wastewater would require WWTP to have longer hydraulic retention time and this could enhance the conditions to promote horizontal gene transfer processes enabling the passage of plasmids and transpose encoding antibiotic resistance (Manaia et al., 2010). Manaia et al. (2010) estimated 1–5% of the total enterobacteria species were CIP resistant in the treated wastewater from domestic WWTPs. For FQ, the induction of the SOS repair response system may enhance bacterial survival and could eventually lead to antimicrobial resistance (Cirz et al., 2005; Dörr et al., 2009). Bacteria only needs to be exposed at sub-inhibitory concentrations antibiotics to foster resistance (Kümmerer, 2004).

In the aquatic environment, the fate of CIP is governed by several mechanisms such as photodegradation, adsorption and biotransformation (Cardoza et al., 2005). CIP was reported as not readily biodegradable (Al-Ahmad et al., 1999; Kümmerer et al., 2000). Investigations of lab-scaled photolysis as a treatment method (UV lamp) or as simulated sunlight (Xenon lamp) to degrade CIP has shown the formation of transformation products (TPs) with structures that retained the core quinolone molecule but with alterations, substitutions and/or deletion of its substituents (Chételat et al., 1996; Sánchez et al., 2005; Vasconcelos et al., 2009; Paul et al., 2010; Garcia-Käufer et al., 2012; Haddad and Kümmerer, 2014). Moreover, Haddad and Kümmerer (2014) identified the same photo-TPs in both UV and Xe lamp photolysis. Several *in vitro* genotoxicity assays have shown that the irradiated mixtures containing TPs and CIP may be mutagenic in the Ames test and genotoxic to several cell lines including mouse lymphoma, human hepatic carcinoma cells (HepG2) and human T lymphocyte cells (Jurkat cells) (Chételat et al., 1996; Sánchez et al., 2005; Garcia-Käufer et al., 2012). Even though CIP is a known *umuC* inducer, none of these studies have monitored the changes in genotoxicity of treated CIP using the umu test. However, quantitative structure activity relationships (QSAR) predicted that some TPs may be capable of inducing the *umuC* gene at lower concentrations than CIP (Li et al., 2014).

In this study we monitored the genotoxicity of CIP and its mixture of TPs after UV irradiation as both a whole mixture analysis using a battery of genotoxicity assays (including the umu assay for SOS repair response) and an individual TP analysis with *in silico* predictions using QSAR models. UV light was selected for the photolysis treatment method since it resulted in higher relative abundance of the individual photo-TPs (Haddad and Kümmerer, 2014). This was important since the aims of the study were to determine the genotoxicity of the UV mixtures and to understand the possible genotoxic role of the stable TPs. The design of the study largely followed an effect driven approach for TP assessment as proposed by Escher and Fenner (2011) with recommendations for genotoxicity characterization of TPs from Toolaram et al. (2014).

2. Materials and methods

2.1. Photodegradation and mineralisation monitoring

Photodegradation of CIP (CAS RN: 85721-33-1; from Sigma–Aldrich) was performed in a 1L immersion-type reactor (UV-Consulting Peschl) using a 150 W medium-pressure mercury lamp (TQ 150, UV-Consulting Peschl, Text S1). The reactor was filled with CIP solution in Millipore water (20 mg/L) and irradiated for 128 min. The initial CIP concentration was selected based on the detection limits for non-purgeable organic carbon (NPOC) measurements using a Shimadzu TOC-5000 analyser. Further information on experimental-setup can be found in Haddad and Kümmerer (2014).

2.2. Liquid chromatography analysis

Detection, identification and quantification, of CIP and its TPs were performed on Agilent Technologies 1100 HPLC series connected to a mass spectrometer Bruker Daltonics Esquire 6000 ^{Plus} equipped with an atmospheric pressure electrospray ionization (AP-ESI) source. Chromatographic Separation was performed on a RP18 EC 125 mm × 4 mm, 5 μm Nucleodur reverse phase column (Macherey–Nagel). Additionally, the accurate masses of CIP and its TPs were measured by LTQ-Orbitrap XL mass spectrometer interfaced with a heated electrospray ionization (H-ESI) source (Thermo Scientific). All LC instruments, chromatographic parameters and mass spectrometer settings have been detailed in Haddad and Kümmerer (2014).

2.3. QSAR predictions

Structure illustrations were performed with MarvinSketch 5.8.0. using simplified molecular input line entry specification (SMILES) codes. These SMILES codes were introduced into various computer based QSAR models for predicting the effects on a number of genotoxicity endpoints.

In silico toxicity predictions of CIP and its TPs were performed using a set of QSAR software each with different algorithms and training sets. The software included CASE Ultra V.1.4.6.6 (MultiCASE Inc.) (Saiakhov et al., 2013), and Leadscope software V.3.0.11–1 with training sets from 2012 SAR Genetox Database (Leadscope) (Roberts et al., 2000). Also Oasis Catalogic software from Laboratory of Mathematical Chemistry (University Bourgas, Bulgaria) predicted mutagenicity based on bacterial mutagenicity (module mutagenicity v.04) in *Salmonella typhimurium* (*Salmonella* Catalogic model, SC).

All *in silico* models had validated database and training sets (Roberts et al., 2000; Chakravarti et al., 2012; Saiakhov et al., 2013). Further information on each model can be seen in Supplementary (Table S1). These models also have been applied in other works (Mahmoud et al., 2014; Rastogi et al., 2014).

2.4. Genotoxicity testing

Prior to testing, samples were kept at 4 °C for 24 h to reduce the presence of short lived reactive oxygen species that can affect the bioassays (Vasquez et al., 2013) and to ensure that mostly stable transformation products were considered in the mixtures. Then the samples were sterile filtered (0.2 μm) and frozen in aliquots at −150 °C. All tests were performed at least twice with 3 replicates per bacterial test and 2 replicates for the *in vitro* MN test. Sample pH