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Transformation products in the water cycle and the unsolved problem of their proactive assessment: A combined *in vitro*/*in silico* approach

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ABSTRACT

Transformation products (TPs) emerging from incomplete degradation of micropollutants in aquatic systems can retain the biological activity of the parent compound, or may even possess new unexpected toxic properties. The chemical identities of these substances remain largely unknown, and consequently, the risks caused by their presence in the water cycle cannot be assessed thoroughly. In this study, a combined approach for the proactive identification of hazardous elements in the chemical structures of TPs, comprising analytical, bioanalytical and computational methods, was assessed by the example of the pharmaceutically active micropollutant propranolol (PPL). PPL was photo-transformed using ultraviolet (UV) irradiation and 115 newly formed TPs were monitored in the reaction mixtures by LC-MS analysis. The reaction mixtures were screened for emerging effects using a battery of *in vitro* bioassays and the occurrence of cytotoxic and mutagenic activities in bacteria was found to be significantly correlated with the occurrence of specific TPs during the treatment process. The follow-up analysis of structure-activity-relationships further illustrated that only small chemical transformations, such as the hydroxylation or the oxidative opening of an aromatic ring system, could substantially alter the biological effects of micropollutants in aquatic systems. In conclusion, more efforts should be made to prevent the occurrence and transformation of micropollutants in the water cycle and to identify the principal degradation pathways leading to their toxicological activation. With regard to the latter, the judicious combination of bioanalytical and computational tools represents an appealing approach that should be developed further.

1. Introduction

Due to the ubiquitous presence in the water cycle, micropollutants can undergo different biotic and abiotic transformation processes along their lifecycle that often lead to the formation of uncharacterized transformation products (TPs). In fact, the presence of numerous active pharmaceutical ingredients along with some of their known TPs in surface water and ground water was recently reported (López-Serna et al., 2012, 2013). However, the chemical identities of most TPs in the aquatic cycle remain still unknown, and therefore, the risk due to their presence cannot be assessed thoroughly (Evgenidou et al., 2015; Fatta-Kassinos et al., 2011; Kosjek and Heath, 2008; Zwiener, 2007). In response to this problem, the proactive assessment of TPs, *i.e.* the characterization of degradation products even before the parent compound comes into use, was repeatedly demanded to be more consequently implemented into the existing regulations for marketing authorization of chemical products (*e.g.* Fenner et al., 2002; Schmitt-Jansen et al., 2007).

The transformation of micropollutants in the aquatic environment is triggered by hydrolysis, photochemical reactions and microbial processes (Längin et al., 2008; Kümmerer, 2008; Packer et al., 2003; Arnold and McNeill, 2007). Beyond that, some water treatment processes, such as the treatment of wastewater using advanced oxidation processes (AOPs) or the disinfection of drinking water by chlorination, ozonation or ultraviolet (UV) irradiation, are well-established pathways for the emergence of TPs (Canonica et al., 2008; Kümmerer et al., 2016). It is known that environmental behavior and hazardous properties of TPs can strongly differ from the parent compounds (DellaGreca et al., 2003; Escher and Fenner, 2011; Garcia-Käufer et al., 2012; Li et al., 2016; Schulze et al., 2010). Moreover, there are strong indications that TPs of pharmaceuticals can remain pharmacologically active (Halling-Sørensen et al., 2002; Rastogi et al., 2015). As a consequence, different strategies for the identification of potentially hazardous TPs

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Abbreviations: 4-OH PPL, 4-Hydroxypropranolol; 5-OH PPL, 5-Hydroxypropranolol; 7-OH PPL, 7-Hydroxypropranolol; AOP, Advanced oxidation process; ANOVA, Analysis of variance; DAD, Diode array detector; EDA, Effect-directed analysis; ESI-IT-MS, Electrospray ionization ion trap mass spectrometry; IR, Induction ratio; LC-MS, Liquid chromatography-mass spectrometry; LBT, Luminescent bacteria test; NPOC, Non-purgeable organic carbon; PPL, Propranolol; (Q)SAR, (Quantitative) structure-activity-relationships; ThEL, Theoretical exposure level; TP, Transformation product; UV, Ultraviolet.

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have been suggested and applied in the recent past. These approaches can be broadly categorized as exposure-driven or effect-driven and have already been extensively reviewed elsewhere (*e.g.* Escher and Fenner, 2011; Fatta-Kassinos et al., 2011; Toolaram et al., 2014; Zonja et al., 2014).

The comprehensive risk assessment of TPs is still impeded by the current lack of standardization in the experimental design (Fatta-Kassinos et al., 2011; Toolaram et al., 2014). Moreover, only in a few cases was it possible to clearly attribute the toxicity of treated mixtures to any of the identified TPs (Escher and Fenner, 2011; Fatta-Kassinos et al., 2011). This was mainly achieved by the coupling of laboratory-scale treatment processes with effect-directed analysis (EDA), which is usually based on a combination of biotesting, fractionation procedures and chemical analytical methods (Brack, 2003). As an example, several toxic photoproducts of anthracene were identified using simulated sunlight irradiation in combination with EDA (Brack et al., 2003). In another study, EDA was used to identify a phytotoxic photo-transformation product of diclofenac (Schulze et al., 2010). Moreover, the toxicity of TPs in treated mixtures was assessed by correlating the time course of the relative TP concentration with the observed mixture effect during the experiment. Using this simple screening method, phytotoxic TPs were tentatively identified in an irradiated mixture of diclofenac (Schmitt-Jansen et al., 2007).

Even if a toxic TP can be successfully identified in a treated mixture, additional research is necessary to elucidate the responsible chemical structural elements and reaction pathways. (Quantitative) structure-activity-relationships ((Q)SARs) are increasingly used to identify hazardous structural features *in silico*, especially in cases where chemicals are not experimentally accessible, which holds true for most TPs. Consequently, (Q)SAR methodologies were recently suggested to be used for the risk assessment of pharmaceuticals and their TPs (Rastogi et al., 2014; Toolaram et al., 2014). Moreover, the recently established ICH M7 guidelines for the assessment of DNA reactive impurities in pharmaceuticals represent a significant advancement in the regulatory acceptance of (Q)SAR models (Barber et al., 2015). These recent developments could indicate new ways for the assessment and regulation of TPs in the water cycle.

In the light of the aspects discussed above, a combined approach for the proactive identification of hazardous elements in the chemical structures of TPs, comprising basic analytical, bioanalytical and computational methods, was explored by the example of the pharmaceutically active micropollutant propranolol (PPL). The nonselective beta-adrenergic receptor-blocking agent was chosen as a model compound for the following reasons:

i. PPL was detected at concentrations up to 373 ng L^{-1} in WWTP effluents, up to 590 ng L^{-1} in river water, and at average concentrations of 1.8 ng L^{-1} in ground waters (Kostich et al., 2014; Santos et al., 2010; Ternes, 1998; Vulliet and Cren-Olivé, 2011).
ii. The presence in ground water of 4-hydroxypropranolol (4-OH PPL), a pharmacologically active human metabolite and TP of PPL, was recently reported (López-Serna et al., 2013).
iii. PPL was reportedly susceptible to phototransformation during technical treatment processes using UV light and also at environmentally relevant conditions using sunlight or simulated sunlight (Andreozzi et al., 2003; Dantas et al., 2010; Liu and Williams, 2007; Peng et al., 2014).
iv. The photochemical reactions of PPL have been intensely researched and with that the formation of a large number of TPs with mainly unknown properties was revealed (Liu and Williams, 2007; Rastogi et al., 2015; Santiago-Morales et al., 2013; Sortino et al., 2002).

The few available studies on the biological effects that could emerge from transformation of PPL do not allow a concluding evaluation of the responsible degradation pathways. Liu et al. (2009b) showed a reduction of ecotoxicity during simulated sunlight-driven photolysis of 10 mg L^{-1} PPL using bioassays with algae and rotifers. Similarly, irradiation of 25 mg L^{-1} PPL with simulated sunlight resulted in a moderate reduction of the cytotoxicity in bacteria (Santiago-Morales et al., 2013). In contrast, Peng et al. (2014) found evidence for the occurrence of intermediate products with increased cytotoxicity in bacteria during UV photolysis of PPL, but the chemical identity of these TPs was not further elucidated. Interestingly, an increasing toxicity to bacteria was also reported after ozonation of PPL at 100 mg L^{-1} and this time three intermediate TPs (m/z $[M + H]^+$ 292, 266 and 282) were identified in the reaction mixture (Dantas et al., 2011). Moreover, it was found that PPL had the ability to induce hemolysis of red blood cells and DNA cleavage upon UVA irradiation (Sortino et al., 2002). Consequently, there are strong indications for the emergence of cytotoxic and genotoxic TPs during UV photolysis of PPL, but there is still insufficient knowledge on the underlying structure-activity-relationships. This impedes the deduction of principle transformation pathways for the toxicological activation of PPL and other related compounds.

This study was not designed to accurately simulate the fate of a compound under specific conditions, but rather (i) to obtain new information on the principle reaction pathways possibly leading to the emergence of hazardous TPs in aquatic environments and (ii) to assess the additional value of a combined *in vitro*/*in silico* toxicity approach to the proactive identification of potentially hazardous TPs. In order to attain these objectives, a synthetic mixture of TPs was generated from PPL using UV irradiation. This reaction mixture was screened for potentially hazardous TPs using liquid chromatography-mass spectrometry (LC-MS) in combination with selected *in vitro* bioassays for cytotoxic and genotoxic activities. Finally, established (Q)SAR models were used for the *in silico* analysis of structure-activity-relationships in order to attribute the observed effects to specific chemical structure elements.

2. Material and methods

2.1. Chemicals

(±)-Propranolol hydrochloride (CAS 318-96-9) was purchased from Sigma-Aldrich (St. Louis, USA). (±)-4-Hydroxypropranolol hydrochloride (CAS 10476-53-6), (±)-5-hydroxypropranolol hydrochloride (CAS 62117-35-5) and (±)-7-hydroxypropranolol (CAS 81907-81-5) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, USA). All solutions were prepared in ultrapure water.

2.2. Photolysis of propranolol

A high initial concentration of 338 μmol L^{-1} (100 mg L^{-1}) PPL hydrochloride was used for the characterization of TPs and the determination of organic carbon removal, taking into account the limited sensitivity of the analytical instruments and the selected bioassays. An additional experiment was conducted with an optically dilute solution (3.4 μmol L^{-1}) to determine the quantum yield (Φ) and to confirm that principal degradation pathways were not altered by the usage of a high initial concentration. The photolysis was conducted with an initial volume of 800 mL using a medium pressure mercury lamp (TQ150, UV Consulting Peschl, Mainz, Germany) in a cylindrical immersion-type batch reactor with an ilmasil quartz immersion tube. The reaction mixture was stirred constantly and the temperature was maintained between 17 and 20 °C. A sampling volume of 20 mL was collected before the treatment (0 min) and after 2, 4, 8, 16, 32, 64, 128 and 256 min of irradiation. The pH and the peroxide concentration in the photolysis mixture were monitored immediately after sampling. Peroxide was determined semi-quantitatively using MQuant™ peroxide test strips (Merck KGaA, Darmstadt, Germany). The non-purgeable organic carbon (NPOC) was measured directly after the treatment process using a Shimadzu TOC-VCPN analyzer. The photolysis samples for chromatographic analysis and *in vitro* testing were initially stored at −20 °C