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A Toxicological Study on Photo-degradation Products of Environmental Ibuprofen: Ecological and Human Health Implications

Nishanthi Ellepola^a, Talysa Ogas^a, Rubi Gurung^a, Danielle N. Turner^b, Sabino Maldonado-Torres^a, Rodolfo Tello-Aburto^a, Praveen L. Patidar^a, Snezna Rogelj^b, Menake Piyasena^a, Gayan Rubasinghege^{a,*}

^aDepartment of Chemistry, New Mexico Institute of Mining and Technology, Socorro, NM 87801

^bDepartment of Biology, New Mexico Institute of Mining and Technology, Socorro, NM 87801

Abstract

Increasing quantities of pharmaceutical waste in the environment have disrupted the balance of ecosystems, and may have subsequent effects on human health. Although a handful of previous studies have shown the impacts of pharmaceutically active compounds on the environment, the toxicological effects of their degradation products remain largely unknown. In the current study, the photo-degradation products of environmental ibuprofen were assessed for both ecotoxicological and human health effects using a series of *in vitro* assays. Here, six of the major degradation products are synthesized with high purity (>98 %) and characterized with ¹HNMR, ¹³CNMR, FT-IR and HRMS. To evaluate human health effects, three gut microbiota species, *Lactobacillus acidophilus*, *Enterococcus faecalis* and *Escherichia coli*, and two human cell lines, HEK293T and HepG2, are exposed to various concentrations of ibuprofen and its degradation products. On *L. acidophilus*, the ibuprofen degradation product (±)-(2*R*,3*R*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol shows a greater toxic effect while ibuprofen enhances its growth at lower concentrations. At higher concentrations, ibuprofen shows at least a 2-fold higher toxicity compared to that of its degradation products. However, *E. faecalis* shows little or no effect upon exposure to these compounds. An induction of the SOS response in *E. coli* is observed but limited to only ibuprofen and 4-acetylbenzoic acid. In human cell line studies, survival of both HEK293T and HepG2 cell lines is profoundly impaired by the photo-degradation products of (±)-(2*R*,3*R*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol, (±)-(2*R*,3*S*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol, and (±)-1-(4-(1-hydroxy-2methylpropyl)phenyl)ethan-1-one. In this work, the bioluminescence bacterium, *Aliivibrio fischeri*, is used as a model to assess environmental impact. Both ibuprofen and its degradation products inhibit the growth of this gram-negative bacteria with the primary compound showing the most significant impact. Overall, our results highlight that

* Corresponding author: gayan.rubasinghege@nmt.edu.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary Material

Supplementary data related to this article is included complete preparation procedures for all the synthesized compounds, characterization data for the synthesized compounds and graphs for all the biological assays with all tested compounds.

some of the degradation products of ibuprofen can be more toxic to human kidney cell line and liver cell line than the parent compound while ibuprofen can be more toxic to human gut microbiota and *A. fischeri* than ibuprofen degradation products.

Graphical Abstract

Keywords

pharmaceuticals; degradation products; gut microbiota; cytotoxicity; ecotoxicity

1. Introduction

The release of large amounts of pharmaceutical waste into the environment has caused an imbalance on our ecosystems, and has recently caused a significant concern for the effects on human health. Ibuprofen, one of the most consumed non-steroidal anti-inflammatory drugs (NSAIDs) around the world, has raised major health concerns due to its ubiquitous use and its subsequent release into the environment (Brozinski et al., 2013; Kristensen et al., 2018; Prud'homme et al., 2017). The hydrophilic nature and weak organic acid properties of ibuprofen play a role in its release from the waste water treatment process to surface water, and account for its presence in our drinking water and tap water in the range of ng/L levels (Carmona et al., 2014; Madikizela and Chimuka, 2017; Pais and Nascimento, 2018). The continual discharge of ibuprofen and its short life-time in water collectively contribute to the phenomena called “pseudo persistence” of this pharmaceutical in the surface water (Amariei et al., 2017; Gonzalez-Mira et al., 2018; Maldonado-Torres et al., 2018; Mezzelani et al., 2018; Prud'homme et al., 2018) and showing up at the detection limit of ibuprofen in natural water bodies in the µg/L range (Prud'homme et al., 2018).

The large amounts of pharmaceuticals present in the environment could be associated with a wide array of adverse effects on human health. The primary ingestion of pharmaceuticals can occur via drinking water, and bioaccumulation in food items grown on biosolids and effluents (Al-Farsi et al., 2017; Brown et al., 2019; Carmona et al., 2014; Pais and Nascimento, 2018). Thus, the main organs affected by pharmaceuticals can be gut, kidney and liver. Investigators suggest that NSAIDs, such as ibuprofen and aspirin, can alter the intestinal microbial composition over long-term consumption (Weiss and Henne, 2017). Conversely, it has been reported that acute kidney injury is a global health concern (Lazzeri et al., 2018; van Koppen et al., 2012), and that renal complications have been observed in

NSAIDs exposed individuals (Arthur et al., 2018; Komhoff et al., 1997; Whelton et al., 2000). The liver is equally important in terms of biotransformation of exogenous compounds (Gonzalez-Ponce et al., 2018). Impairment of both the liver and kidney can disrupt the metabolic homeostasis of the human body. Moreover, the anti-inflammatory effect of ibuprofen is due to the inhibition of cyclooxygenase (COX) enzymes that make prostaglandins (Vane and Botting, 1996; Vane and Botting, 1998). A recent study reported that in addition of the inhibition of the COX enzymes, ibuprofen can also alter the endocrine system and induce compensated hypogonadism in men; revealing further risk of pharmaceuticals in the environment on human health (Kristensen et al., 2018). However, it is noteworthy to mention that, to date, there is no evidence to date that the low concentrations of pharmaceuticals present in drinking water are having adverse effects on the human gut, kidney or liver. The current study thus only focuses on the potential toxicological impact on the above organs via a series of *in vitro* assays. Our findings will reveal a new topic for future investigations, highlighting challenges for toxicologists to explore the effects of pharmaceutical waste on human health.

The widespread occurrence of these pharmaceuticals in the natural environment is often a concern due to their designed biologically-active nature, as such, they may have potential impact on non-targeted organisms along with other environmental stresses such as heavy metals, pesticides, organic and inorganic compounds (Aguirre-Martinez et al., 2013b). The significant reduction of lysosomal membrane stability after exposure to ibuprofen in *Ruditaphes philippinarum* (Aguirre-Martinez et al., 2013b), *Carcinus maenas* (Aguirre-Martinez et al., 2013a) and *Mytella charruana* (Pusceddu et al., 2018) highlights its potential risk on non-targeted organisms. Similarly, the presence of ibuprofen in the environment can inhibit the bioluminescence of *Aliivibrio fischeri*, a marine bacteria, at concentrations ranging from 36–164 ng/g level (Pusceddu et al., 2018). Given that *A. fischeri* is a well-known for its natural symbiosis with Hawaiian bobtail squid, (Bongrand and Ruby, 2018; Ruby et al., 2005), any harmful effect on its bioluminescence can lead to ecosystem imbalance over time. Collectively, this evidence agrees with the hypothesis that ubiquitous presence of ibuprofen is a crucial environmental issue.

Moreover, ibuprofen may exist in open water bodies not only in the form of the parent compound, but also as its degradation products, produced by various environmental conditions (Arthur et al., 2018; Jallouli et al., 2018). Surprisingly, the occurrence, the persistence, and the toxicological effects of these degradation products of pharmaceuticals, including ibuprofen, are poorly understood. Several previous studies on micropollutants, including certain pesticides and herbicides, have reported that toxic photodegradation products are generated under UV-treatment (Jiang and Gan, 2016; Souissi et al., 2013). These studies further claim that degradation products are at least as toxic as the parent compounds, and pose a threat to ecosystems and human health. It is also important to mention that although concentrations of some of these individual compounds could be very low in the water systems, it is unknown if the presence of numerous pharmaceutical residues and/or their degradation products sharing a specific effect could compound into significant health effects (Juurlink et al., 2003). Thus, investigating the impact of parent pharmaceutical ibuprofen, as well as its degradation products on human health and environment is of essence.

In our previous work, we reported enhanced degradation rates of ibuprofen in the presence of sunlight and clay particles to yield a mixture of photo-degradation products (Rubasinghege et al., 2018). The major photo-degradation product was 4-acetyl benzoic acid followed by 4-(1-carboxyethyl)benzoic acid, (2*R*,3*R*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol, (2*R*,3*S*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol, 4-(3-hydroxy-2-methylpropanoyl)benzoic acid, 4-isobutyrylbenzoic acid, and 1-(4-(1-hydroxy-2-methylpropyl)phenyl)ethan-1-one. In this follow up study, we focus on the synthesis and biological characterization of these photo-degradation products. Here, six major compounds are successfully synthesized and characterized using ¹H NMR, ¹³CNMR, FTIR and HR-MS. The human health effects of major photo-degradation products are evaluated against their parent compound, ibuprofen, using selected gut microbiota species: *Lactobacillus acidophilus*, *Enterococcus faecalis*, *Escherichia coli* and two human cell lines: HEK293T (Kidney), HepG2 (Liver). The ecotoxicological effects of the degradation products are tested on the Gram-negative bioluminescent marine bacterium: *Aliivibrio fischeri*. The overall results of this study provide a new insight into the potential risk of exposure to ibuprofen and its' photo-degradation products on the human health and environment. Our results highlight that the toxicological effects of the photo-degradation products of ibuprofen are significantly different from its parent compound and that some photo-degradation products are even more toxic than ibuprofen.

2. Materials and Methods

2.1. Experimental Design

The present work was carried out as part of a broader study of ibuprofen photo-degradation products which were identified in our previous work (Rubasinghege et al., 2018). Seven photo-degradation products of ibuprofen were selected based on their relative abundances, and tested against the parent compound ibuprofen. Ibuprofen and its major photo-degradation product, 4-acetyl benzoic acid, were purchased from Sigma Aldrich (Missouri, USA) while all the other six compounds (Figure 1), (±)-4-(1-carboxyethyl)benzoic acid (**1**), (±)-(2*R*,3*R*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol (**2a**), (±)-(2*R*,3*S*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol (**2b**), (±)-4-(3-hydroxy-2-methylpropanoyl)benzoic acid (**3**), 4-isobutyrylbenzoic acid (**4**), and (±)-1-(4-(1-hydroxy-2-methylpropyl)phenyl)ethan-1-one (**5**) were synthesized, and characterized using ¹H NMR, ¹³CNMR, IR and HRMS. The synthesized compounds were stored at -20 °C until use. The biotoxicity and ecotoxicity effects of the above compounds were evaluated using the procedures described below.

All biological experiments were performed in three independent replicates, and each experiment performed in at least triplicate. The results are reported as average ± standard deviation. The relative toxicity is represented as the normalized values as compared to DMSO controls to eliminate any possible solvent effect.

2.2 Synthesis of Ibuprofen Degradation Products

All moisture sensitive reactions were conducted in oven-dried glassware under an atmosphere of dry Argon. Reaction solvents were dried and degassed by passing through a

column of activated alumina in a solvent purification system or freshly distilled from the appropriate drying agent. All other solvents and reagents were purchased from commercial suppliers and used as received, unless otherwise specified. Isolated yields refer to chromatographically homogeneous material, and are not optimized.

Compound **1** was synthesized from methyl 2-(4-bromophenyl)acetate, using a cyanation reaction (Zanon et al., 2003), followed by alkylation and hydrolysis. The synthesis of compounds **2a** and **2b** started with ibuprofen and involved reduction of the carboxylic acid, oxidation to form an aldehyde and addition of isobutyl magnesium bromide. This sequence of reactions produced compound **2a** as major product and compound **2b** as minor product contaminated with **2a**. An independent synthesis of compound **2b** was thus required and started with **2a**. Oxidation of **2a** to the corresponding ketone and reduction gave the compound **2b** as the major product. The synthesis of compound **3** used 4-formylbenzoic acid as the starting material, and started by formation of the corresponding methyl ester. Addition of ethyl magnesium bromide, followed by oxidation of the resulting secondary alcohol to the corresponding ketone, aldol reaction with formaldehyde, and final hydrolysis then delivered compound **3**. The synthesis of compound **4** also started with 4-formylbenzoic acid. The sequence involved formation of a benzyl ester, addition of isopropyl magnesium chloride and oxidation of the resulting benzylic alcohol to the ketone followed by hydrolysis. Compound **5** was prepared from benzyl 4-(1-hydroxy-2-methylpropyl)benzoate by hydrogenolysis and direct conversion of the resulting carboxylic acid into the corresponding methyl ketone **5** (Genna and Posner, 2011). Detailed descriptions of all synthetic procedures are provided in the Supporting Information. All final compounds and intermediates were characterized by spectroscopic methods: NMR, IR and HRMS.

All the synthesized compounds were subjected to HPLC analysis (Agilent HPLC 1200 series, Eclipse Plus C18 Column, UV detector) to determine the purity before any biological testing carried out. The HPLC analyses were performed at isocratic mode with mobile phase 60 % acetonitrile and 40 % sodium phosphate buffer at 1 mL/min flow rate. Wave length of the UV detector was set to 220 nm.

2.3. Biological Assay

2.3.1. Bacterial Strains and Growth Conditions—Glycerol stocks of RecA-GFP-expressing *Escherichia coli* (MG1655; RecA) and *E. faecalis* (ATCC 51299 (VRE)) were stored at -80°C . *E. coli* was cultured in Mueller-Hinton broth (MHB) plus 10 $\mu\text{g/mL}$ kanamycin at 37°C for 12 hours to produce an overnight growth. *E. faecalis* was grown in MHB for 12 hours at 37°C . A stock of freeze-dried *L. acidophilus* (ATCC-4356) was stored at 4°C . A culture of *L. acidophilus* was made by inoculating Lactobacilli MRS Broth (LMRSB) and incubating at 37°C in an anaerobic chamber for 24 hours.

2.3.2. In vitro Compound Screening—To assess the biotoxicity, ibuprofen, 4-acetyl benzoic acid, ($\pm$)-4-(1-carboxyethyl)benzoic acid (**1**), ($\pm$)-(*2R,3R*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol (**2a**), ($\pm$)-(*2R,3S*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol (**2b**), ($\pm$)-4-(3-hydroxy-2-methylpropanoyl)benzoic acid (**3**), 4-isobutyrylbenzoic acid (**4**), and ($\pm$)-1-(4-(1-hydroxy-2-methylpropyl)phenyl)ethan-1-one (**5**) were dissolved in DMSO at a concentration

of 100 mM. All compounds were screened for toxicity in 96-well plates to which 100 μ L of 5×10^5 CFU/mL of *E. faecalis* or *L. acidophilus* had been added. Compounds were screened at a starting concentration of 100 μ M on *E. faecalis* and *L. acidophilus*, and two-fold serial dilutions of the compounds were made. Wells containing MHB or LMRSB, and non-treated cells served as negative controls. Wells containing 1 % v/v DMSO served as vehicle controls while wells containing 100 μ M phenylarsine oxide (PAO) treated cells served as positive controls. The plates containing *E. faecalis* were incubated in a rotary shaking incubator for 18 hours at 37 °C. Plates containing *L. acidophilus* were incubated in a stationary incubator at 37 °C with 5 % CO₂ for 24 hours. Compound activity was assessed by an MTT viability assay for *E. faecalis*, whereby 20 % w/v MTT (5 mg/mL stock) was added to each well. The plates were incubated for 20 minutes at 37 °C. 150 μ L of solubilization solution (50 % volume of 20 % SDS and 50 % volume of dimethylformamide) was added to each well, and the absorbance was measured at 595 nm. The compound activity against *L. acidophilus* was assessed by measuring absorbance at 600 nm to determine cell density.

2.3.3. Screening for Genotoxicity via Activation of *E. coli* SOS Response—An overnight growth of RecA-GFP-expressing *E. coli* (MG1655; RecA) was diluted to 1×10^7 CFU/mL in MHB plus 10 μ g/mL kanamycin. Compounds were screened for genotoxicity in a 24-well plate to which 500 μ L of *E. coli* had been added. All compounds were screened at concentrations of 100 μ M and 10 μ M. Wells containing non-treated cells served as negative controls, wells containing 1 % v/v DMSO served as vehicle controls, and wells containing 0.19 μ M norfloxacin served as positive DNA-damage-inducing controls. The plates were incubated for 4 hours at 37 °C before being analyzed via flow cytometry for the induction of the bacterial SOS response which was displayed by the production of green fluorescence.

2.3.4. Cell Culture and Cell Viability Assays—Cell lines HEK293T and HepG2 were obtained from American Culture Type Collection (ATCC, Manassas, VA), and were grown in DMEM (Dulbecco's Modified Eagle Medium from Lonza, Walkersville, MD) containing 10 % FBS (Fetal Bovine Serum) in humidified 5 % CO₂-95 % air atmosphere at 37 °C. Cells were seeded in 96-well plates (4,000 cells/well) and allowed to adhere overnight. The next day, cells were treated in fresh medium with 250 μ M, 125 μ M, and 62.5 μ M concentrations of ibuprofen or its photo-degradation products for 48 hours. Phenylarsine oxide (PAO, Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 10 μ M was used as a positive control, and 0.5 % dimethyl sulfoxide (DMSO, Sigma-Aldrich) was used as a negative control. After 48 hours of incubation, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) solution (5 mg/mL in 1 $\times$ PBS) was added to each well, and cells were incubated at 37 °C for 2 hours. Subsequently, the supernatants were aspirated, and 100 μ L of 100 % DMSO was added to each well. Absorbance was then measured at 570 nm using a coulter microplate reader. Data (% mean $\pm$ S.D.) were expressed as treated/control (i.e., ibuprofen or its photo-degradation products/DMSO) values from at least three biological replicates each performed in at least triplicate. IC₅₀ values were calculated from cell viability data using Microsoft Excel.

2.3.5. Preparation of *A. fischeri* Culture and Test Chemicals—*Aliivibrio fischeri* (ATCC® 7744™) was selected for all the bioluminescence assays because it is simple, fast,

inexpensive, and highly sensitive model that has been employed in numerous previous studies (Jarque et al., 2016). The bacterial culture was grown in photobacterium growth medium (pH = 6.94) purchased from Sigma Aldrich (Missouri, USA). The culture was incubated in a temperature-controlled incubating shaker (VWR, Pennsylvania, USA) at 25 °C with gentle shaking. Significant bacterial growth and constant luminescence were observed after 24 hours of incubation; therefore, bacterial culture during this period was selected for the toxicity assays. 100 mM stock solutions of the test chemicals were prepared in DMSO, and were used for the bioluminescence inhibition assays.

2.3.6. Acute Toxicity Testing through Bioluminescence Inhibition of *A. fischeri*

The toxicity assay was performed in a 96-well plate, with the initial drug concentration of 1 mM in the first well, followed by a 1:2 serial dilution. DMSO control was first analyzed to evaluate the solvent effect on bioluminescence of *A. fischeri*. Phenylarsine oxide (PAO) was selected as a positive control for toxicity with the final test concentration of 330 µM. The final test concentrations of the test chemicals were 500 µM, 250 µM, and 125 µM.

Bacterial luminescence was monitored using Infinite M Plex microplate reader (TECAN, Switzerland) at 15 minutes. Black 96-well plates (Greiner Bio-One, Austria) were used for all luminescence measurements. The relative toxicity was calculated by normalizing the luminescence of the treated sample to that of the control as shown in, Eq. 1.

$$\text{Relative Toxicity} = \frac{\text{Luminescence of the treated sample}}{\text{Luminescence of the control}} \times 100 \quad \text{Eq.1}$$

2.4 Statistical analysis

The statistical analysis was conducted using Minitab Express. It should be pointed out that these toxicological studies were mainly focused on evaluating health effects of primary ibuprofen and its degradation products relative to each other. The two-way ANOVA followed by the Student's t-test were used to identify significant differences among the toxicological effects of ibuprofen and its photo-degradation products on selected microbiota and human cell lines, and to reveal differences between control and treated groups of various concentrations. Unless otherwise noted, the differences were considered statistically significant if $p \leq 0.05$.

3. Results and Discussion

3.1 Synthesis

All the synthesized structures of photo-degradation products of ibuprofen were confirmed by ¹HNMR, ¹³CNMR, FT-IR and HRMS. These results are presented in Supplementary Information. Table: 1 summarizes the purity of all the compounds used in the biological assay, which had above 98 % purity or better, and was further confirmed by HPLC.

3.2 Toxicological Studies

The studies aimed to evaluate the relative toxicity of ibuprofen and its photo-degradation products on human health and environmental organisms. *In vitro* biotoxicological studies were carried out with three gut microbiota species, *Escherichia coli*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, and two human cell lines, Hek293T (kidney) and HepG2 (liver). Ecotoxicological effect was assessed using a bioluminescent marine bacterium *Aliivibrio fischeri*. In this study, the DMSO-treated group served as a vehicle control and phenylarsine oxide (PAO)-treated group served as a positive control where the entire population of the cells was dead. The samples were then evaluated and reported as normalized cell survival $\pm$ standard deviation. Results with the most significant toxic effects are shown in the main article while the complete dataset is provided in the Supplemental Information.

It is also important to point out that the concentration of ibuprofen used in the current work is higher than its reported concentration in the environment; this due minimum sensitivity limitation of our established toxicological assays. These concentrations also vary slightly among our different biological assays due to the differences in assay sensitivities. The reported environmental concentration of ibuprofen ranges from 50 to 250 nM (Fekadu et al., 2019; Khazri et al., 2019), while no field measurements are available for the degradation products. As reported in our previous work (Rubasinghege et al., 2018), ibuprofen degrades at an accelerated rate in the presence of clay under solar radiation, resulting in a shortened lifetime of primary compounds in water bodies. Thus, the “actual” amount of ibuprofen added to the environment can be higher than the previously reported values. As a consequence, the quantity of secondary residues found in the environment could also be high. However, it is also important to mention that our preliminary experiments, performed with ibuprofen concentrations closer to the reported values, yielded degraded products similar to those found with higher concentrations of ibuprofen. Moreover, these primary and degraded products are expected to further accumulate in the water bodies via biotic and abiotic mechanisms, resulting in higher concentrations yet (Al-Farsi et al., 2018; Muir et al., 2017). Thus, the implications discussed in this work are transferable to the environmentally-relevant conditions.

3.2.1 Human Health effects

3.2.1.1 *In vitro* Screening of Ibuprofen and Photo-degradation Products on Gut

Microbiota Species: Effects of exposure to 100 μ M, 50 μ M, and 25 μ M ibuprofen and its photo-degradation products on gut microbiota species *L. acidophilus* and *E. faecalis*, were investigated. As summarized in Figure 2A, our results indicated that parent pharmaceutical ibuprofen has a statistically significant effect (73% inhibition) on survival of *L. acidophilus* cells compared to DMSO-treated control group at 100 μ M. This data is in agreement with a previous clinical study examining the effect of several NSAIDs, including ibuprofen, indomethacin, diclofenac, ketoprofen, celecoxib, piroxicam, aspirin, and nimesulid, on the gut microbiota of elderly individuals in which they reported a decrease in the survival of *Lactobacillus* genus (Makivuokko et al., 2010). Furthermore, a substantial decrease of *Lactobacillus* sp. has been observed in rats treated with diclofenac, another COX inhibitor similar to ibuprofen (Colucci et al., 2018). However, it should be noted that the 100 μ M used in this work and the ibuprofen doses used in previous clinical studies are at considerably

higher concentrations than reported values for drinking water. Photo-degradation products of ibuprofen in Figure 2A show that all three tested concentrations of compound **2a** killed *L. acidophilus* with a maximum inhibition of 34 % at 100 μ M. Photo-degradation compound **4AB** showed 27 % inhibition at 100 μ M. None of the other compounds showed significant inhibition ($p > 0.05$). As pointed out previously, mammals have a stable and genetically rich gut microbiota that plays an essential role in digestion and metabolism (Shen et al., 2013). For instance, gut microbiota regulate the brain-gut axis via their ability to synthesize and release different neurotransmitters and neuromodulators (Alkasir et al., 2017). In addition, *Lactobacillus sp.* can synthesize short chain fatty acids and the neurotransmitter acetylcholine (Alkasir et al., 2017). It has been postulated that dysfunction in the brain-gut axis can cause irritable bowel syndrome and inflammatory bowel disease (Alkasir et al., 2017); this matches the data that a *Lactobacillus* deficiency can develop NSAIDs-enteropathy (Colucci et al., 2018). Gut microbiota can further act as a barrier for infections (Kamada et al., 2013; Round and Mazmanian, 2009), provide immunological benefits to the host (Kamada et al., 2013; Round and Mazmanian, 2009), as well as regulate the body weight (Ramakrishna, 2013; Shen et al., 2013). In certain cases, the breakdown of the balance between gut microbiota and the host can be detrimental to the host (Shen et al., 2013). The decreased cell survival of *L. acidophilus* demonstrated in Figure 2A might shift the gut microbe balance over time. This would lead to metabolic disturbances in any of above explained metabolic pathways. Further investigations are needed to assess the most plausible consequences.

Despite the toxicological effects on the Gram-positive *L. acidophilus*, no significant effect ($p > 0.05$) on the survival of *E. faecalis* was observed under all tested concentrations of ibuprofen or any of its photo-degradation products (Figure 2B). The increased cell growth of *E. faecalis* upon exposure to compound **4** and **5** may be due to hormesis, a biphasic dose-response phenomenon (Iavicoli et al., 2018). However, this effect was not significant. Although we did not observed any acute toxicity of these compounds in *E. faecalis*, a higher concentration and prolonged exposure over many generations may result in further toxicity. The comparative analysis of gut microbiota carried out in this study further point to new directions in human health implications, caused by pharmaceuticals and their degradation products present in the environment.

3.2.1.2 Screening for Genotoxicity via Activation of *E. coli* SOS Response: SOS is an error-prone response to DNA damage (Wurihan et al., 2018), which can subsequently increase the probability of acquisition of mutations in genome (McCloskey et al., 2018). To investigate whether the above compounds are capable of inducing DNA damage, we explored the *in vitro* SOS response in the gut microbiota species *E. coli* at concentrations of 100 μ M and 10 μ M. A concentration of 0.19 μ M of norfloxacin, a well-established DNA-damaging antibiotic, served as the positive control. As illustrated in Figure 3, ibuprofen showed an increased SOS response for both tested 100 μ M (14% induction) and 10 μ M (10% induction) concentrations. On the other hand, the 100 μ M concentration of the photo-degraded compound **4AB**, showed a 14% increase in the SOS response. These observations can be positively correlated with the abnormalities of cell cycle in *E. coli*, indicating that these compounds may cause cumulative genotoxic effects over long-term exposure. No

particularly notable induction of SOS response was detected from any other photo-degradation compound at the above-mentioned concentrations. The observed genotoxic effects on *E.coli* (and potentially other bacteria) can alter different interactions among diverse species within, and may potentially result in adverse effects to the host, including the development of antibiotic resistance.

3.2.1.3 Cytotoxicity of Ibuprofen and Ibuprofen Photo-degradation Products: A series of standardized *in vitro* cell viability experiments were performed on the human kidney cell line, HEK293T, and on the liver cell line, HepG2. These cell lines were exposed to concentrations of 250 μ M, 125 μ M, and 62.5 μ M of ibuprofen and its photo-degradation products separately. As depicted in Figure 4A, the photo-degradation product of ibuprofen, compound **2a**, showed a profound inhibitory effect ($p < 0.05$) on proliferation of HEK293T cell line at all three concentrations tested in a dose-dependent manner.

Interestingly, at 250 μ M, compound **2a** killed 95 % of HEK293T cells, showing at least a 3-fold increased inhibitory effect when compared to the parent pharmaceutical ibuprofen. Additionally, compound **2b** also killed 95 % of HEK293T cells with 3-fold enhanced inhibition when compared to ibuprofen at the concentration of 250 μ M. Moreover, photo-degradation product **5** also impaired the HEK293T cell growth significantly (50 % killed) at 250 μ M. In contrast, the parent compound ibuprofen did not show any significant ($p < 0.05$) inhibitory effect on HEK293T cell line at any of the tested concentrations. There are reports of renal toxicity of ibuprofen on human kidney (Balestracci et al., 2015; Farquhar et al., 1999; Modig and Elmstahl, 2018), but ibuprofen did not display cytotoxicity within the parameters and constraints of this study, likely due to the low concentrations used. The most plausible mechanism responsible for the therapeutic effects of NSAIDs, including ibuprofen, is the inhibition of prostaglandin (PG) synthesis which also impacts important renal functions such as renin angiotensin aldosterone system (Murray and Brater, 1993) and PG-mediated vasodilation (Farquhar et al., 1999). However, there is no known predictor for nephrotoxicity of photo-degradation products of ibuprofen, and this is the first time reporting of such toxicity. Although our current work does not focus on the mechanism of nephrotoxicity for degradation products of ibuprofen, we wish to highlight the urgent need for such future studies.

Interestingly, HepG2 cells showed a similar impact as the HEK293T cell line (Figure 4B). All three concentrations of compound **2a** impaired the proliferation of HepG2 cell line in a dose depended manner. Remarkably, 250 μ M concentration of compound **2a** showed an 8-fold greater inhibition of HepG2 cell line compared to ibuprofen, killing 94 % of cells. Similarly, compound **2b** also significantly inhibited cell survival at all three tested concentrations, showing a 7-fold inhibition compared to ibuprofen at 250 μ M (88 % killed). Compound **5** moderately impaired HepG2 cell survival (36 % killed) at 250 μ M. No significant inhibitory effect was observed for ibuprofen on the survival of HepG2 cells, even at the highest concentration. Inhibitory mechanisms of ibuprofen or any of the secondary products were not addressed in this study, but are recommended for future studies.

Taken together, these *in vitro* studies confirmed that photo-degradation products of **2a**, **2b** and **5** can significantly damage human liver cells and human kidney cells compared to its

parent compound, ibuprofen. Given the bioaccumulation of these compounds in human tissues and via food webs, the long-term ingestion of degradation products of environmental ibuprofen may potentially impair human liver and kidney function. Outcomes of this study may open the way to identify future environmental health threats due to pharmaceuticals, and their photo-degradation products.

3.2.2 Ecotoxicological Impact - Bioluminescence Based Assay to Determine Acute Toxicity

—Bioluminescence is a well-known energy-dependent (ATP, adenosine triphosphate) process (Fernández-Piñas et al., 2014). Luciferase, the enzyme responsible for producing bioluminescence, catalyzes the oxidation of reduced flavin mononucleotide (FMNH₂) and a long-chain fatty acid aldehyde in aerobic environment. The resulting bioluminescence can be measured at 490 nm (Fernández-Piñas et al., 2014). Any reduction in the luminescence intensity can be attributed to the alteration in bacterial metabolism that results in the change of [ATP], and can therefore be positively correlated to the toxicity imposed by the compounds being tested.

Since the solvent (DMSO) enhanced the bioluminescence of *A. fischeri*, all the data were normalized with respect to the solvent. Figure 5 shows that all three concentrations of parent pharmaceutical ibuprofen, 500 μ M, 250 μ M, and 125 μ M, profoundly diminished the bioluminescence of *A. fischeri* ($p < 0.05$) compared to the DMSO control group. Consistent with previous studies, Figure 5 illustrates that the ibuprofen exhibits the greatest dose-dependent diminution of bioluminescence of *A. fischeri* (Pusceddu et al., 2018). We observed a 60% decrease in bioluminescence in cells treated with 500 μ M of compound. Furthermore, all three concentrations of the tested photo-degradation products showed a significant reduction in bioluminescence, with the exception of compound **4AB** and compound **3**. It has been previously reported that photo-irradiation generated degradation products formed by a mixture of 27 pharmaceuticals, including ibuprofen, showed toxic effects on bioluminescence of *A. fischeri* (Wang and Lin, 2014). To the best of this authors' knowledge, this is the first time reporting on the effects of photo-degradation products of ibuprofen on bioluminescence of *A. fischeri*. As described earlier, the potential negative effects on *A. fischeri* in this study can lead to loss of biodiversity and irreversible ecological changes in the environment. Moreover, highly significant interspecies correlations ($r > 0.9$) have been observed previously for the lethal concentration between *A. fischeri* and many higher order aquatic species, and fairly good correlations ($r \sim 0.75$) with mammals (Kaiser, 1998). Thus, our results from these experiments can be exploited to make confident predictions of toxicity on other species.

4. Conclusion

For the first time, this study evaluates toxic effects of ibuprofen and its photo-degradation products on human cell lines, gut microbiota, and on *A. fischeri*. Our results highlight that the toxicological effects of photo-degradation products of ibuprofen are different from the toxicological effects of its parent compound. Mammalian cell line studies indicate that photo-degradation compounds **2a**, **2b** and **5** impair the proliferation of both HEK293T and HepG2 more than that of ibuprofen, suggesting that degradation products of pharmaceuticals can be more toxic than their parent compounds. On the other hand, ibuprofen was more

toxic to gut microbiota and *A. fischeri* than the photo-degradation products. Additional investigations to elucidate the effects of chronic toxicity and the study of trophic transfer of these products are strongly recommended. Moreover, future studies focused on specific metabolic pathways influenced by these photo-degradation products are suggested to reveal mechanisms behind their different toxic effects.

Finally, our findings from *in vitro* studies confirm that ibuprofen and its photo-degradation products have an adverse effect on human liver cells, kidney cells, gut microbiota species, and *A. fischeri* even over a short-term exposure. The observed toxicological effects can be a major concern for human health due to the increased release of these pharmaceutical wastes into – and their accumulation in – the environment. Here, we suggest future *in vivo* studies to further confirm these toxic effects under more physiological conditions. Moreover, diminishing *A. fischeri* bioluminescence upon exposure to these compounds can lead to ecosystem imbalance over long term. These effects can be spread to other aquatic and terrestrial organisms eventually. Given that these degradation products are capable of yet further transformations and interactions with other pharmaceuticals and their degradation products in natural waters, our work aims to highlight the importance of additional characterization of environmental pollutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Ibuprofen is more toxic to gut bacteria compared to its degradation products.
- Degradation products pose acute toxic effects on human kidney and liver cells.
- Growth of *A. fischeri* is affected by both ibuprofen and its degradation products.

Figure 1.

Structures of synthesized compounds; (±)-4-(1-carboxyethyl)benzoic acid (**1**), (±)-(2*R*,3*R*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol (**2a**), (±)-(2*R*,3*S*)-2-(4-isobutylphenyl)-5-methylhexan-3-ol (**2b**), (±)-4-(3-hydroxy-2-methylpropanoyl)benzoic acid (**3**), 4-isobutyrylbenzoic acid (**4**), (±)-1-(4-(1-hydroxy-2-methylpropyl)phenyl)ethan-1-one (**5**).

Figure 2. Impact of ibuprofen and its photo-degradation products on selected gut microbiota. **A.** *Lactobacillus acidophilus*, **B.** *Enterococcus faecalis*. Data are shown as $\pm$ STD. *Significant ($p < 0.05$), [‡]PAO treated is the positive control where the entire population of the cells was dead, and DMSO-treated is the vehicle control.

Figure 3.

Impact of ibuprofen and its photo-degradation products on DNA damage of *Escherichia coli*.

Data are shown as $\pm$ STD. *Significantly different with respect to the Control ($p < 0.05$), norfloxacin is the positive control (0.19 μ M), DMSO-treated is the vehicle control.

Figure 4.

Impact of ibuprofen and ibuprofen photo-degradation products on proliferation of human cell lines; **A.** HEK293T and **B.** HepG2. Data are shown as $\pm$ STD. * $p < 0.05$, ‡PAO treated is the positive control where the entire population of the cells was dead, DMSO-treated is the vehicle control.

Figure 5. Impact of ibuprofen and its photo-degradation products on bioluminescence of marine bacteria *Aliivibrio fischeri*. Data are shown as $\pm$ STD. *Significant $p < 0.05$, ‡PAO treated is the positive control where the entire population of the cells was dead, DMSO-treated is the vehicle control.

Table 1.

Purity of compounds used in biological studies and acronyms used in the text for the synthesized compounds

Compound	Purity	Acronym
Ibuprofen	99 %	IBU
4-acetylbenzoic acid	98 %	4AB
(±)-4-(1-carboxyethyl)benzoic acid	98.9 %	1
(±)-(2 <i>R</i> ,3 <i>R</i>)-2-(4-isobutylphenyl)-5-methylhexan-3-ol	99 %	2a
(±)-(2 <i>R</i> ,3 <i>S</i>)-2-(4-isobutylphenyl)-5-methylhexan-3-ol	99 %	2b
(±)-4-(3-hydroxy-2-methylpropanoyl)benzoic acid	98 %	3
4-isobutyrylbenzoic acid	99 %	4
(±)-1-(4-(1-hydroxy-2methylpropyl)phenyl)ethan-1-one	99 %	5