ASSESSING THE POTENTIAL OF PHARMACEUTICALS AND THEIR TRANSFORMATION PRODUCTS TO CAUSE MUTAGENIC EFFECTS: IMPLICATIONS FOR GENE EXPRESSION PROFILING

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Abstract: The selection and prioritization of pharmaceuticals and their transformation products for evaluating effects on the environment and human health is a challenging task. One common approach is based on compounds (e.g., mixture composition, concentrations), and another on biology (e.g., relevant endpoint, biological organizational level). Both of these approaches often resemble a Lernaean Hydra—they can create more questions than answers. The present study embraces this complexity, providing an integrated approach toward assessing the potential effects of transformation products of pharmaceuticals by means of mutagenicity, estrogenicity, and differences in the gene expression profiles. Mutagenicity using the tk kinase assay was applied to assess a list of 11 priority pharmaceuticals, namely, atenolol, azithromycin, carbamazepine, diclofenac, ibuprofen, erythromycin, metoprolol, ofloxacin, propranolol, sulfamethoxazole, and trimethoprim. The most mutagenic compounds were found to be β-blockers. In parallel, the photolabile pharmaceuticals were assessed for their mixture effects on mutagenicity (tk assay), estrogenicity (T47D-KB luc assay), and gene expression (microarrays). Interestingly, the mixtures were mutagenic at the μg/L level, indicating a synergistic effect. None of the photolyzed mixtures were statistically significantly estrogenic. Gene expression profiling revealed effects related mainly to certain pathways, those of the p53 gene, mitogen-activated protein kinase, alanine, aspartate, and glutamate metabolism, and translation-related (spliceosome). Fourteen phototransformation products are proposed based on the m/z values found through ultra-performance liquid chromatography–tandem mass spectrometry analysis. The transformation routes of the photolysed mixtures indicate a strong similarity with those obtained for each pharmaceutical separately. This finding reinforces the view that transformation products are to be expected in naturally occurring mixtures.

EXPRESSION PROFILING

INTRODUCTION

Pharmaceuticals are increasingly being used to treat disease and improve health conditions, with the ultimate goal of increasing life expectancy. For example, the consumption of antibiotics increased worldwide by 36% during 2000 to 2010 [1], which raises concerns regarding the potential adverse effects of direct and indirect exposure. Obviously, humans are directly exposed while under treatment or after ingestion or application of a pharmaceutical. Indirect human exposure is related to environmental pollution with contaminants that are not currently monitored.

Pharmaceuticals are released directly into the environment through untreated wastewater, human disposal of unused pharmaceutical products, and animal excretion after veterinary use. Indirectly, they enter through treated urban and industrial wastewater discharges, after their partial elimination in wastewater treatment [2]. As a result, pharmaceutical traces of ng/L to μg/L can be found in the aquatic and terrestrial environment.

After intake, pharmaceuticals are subjected to transformation. They can be biotransformed by the liver and/or the intestinal microbial population [3]. After excretion, further transformation may take place as a result of natural physicochemical and biological processes (photolysis, hydrolysis, deconjugation, biodegradation, and so on) as well as treatment processes (biological, ozonation, ultraviolet [UV] irradiation, and so on). Pharmaceuticals may undergo many transformation steps but rarely reach complete mineralization. For this reason, soils and waters receive not only the parent compounds of pharmaceuticals, but also their metabolites and transformation products [4,5]. The existence of the latter in wastewater and surface water has been already reported at the ng/L level [6,7]. Moreover, it has been shown that some transformation products are of greater concern than parent compounds because of their increased toxicity [8,9].

The processes used to treat drinking water are incapable of completely removing pharmaceuticals, so it is no surprise that traces are found in drinking water as well [10]. Humans can potentially be exposed to pharmaceutical residues by consuming water and food products. To the authors’ knowledge, none of the studies published so far has been able to establish an unambiguous link between risk and indirect human exposure to pharmaceuticals. Even so, the public’s perception regarding water and wastewater reuse is already negatively affected [11].

The environmental and human health concerns about exposure to pharmaceuticals are based on their intrinsic biological potency. In most cases, pharmaceuticals react directly with a specific target molecule, thereby influencing...
its function. Detrimental effects occur when an adverse outcome pathway initiating a chain of causally connected events at different biological organizational levels is activated [12]. In the present study, molecular and cellular endpoints were investigated, with the aim of reaching a better understanding of adverse outcome pathways related to unintended exposure to pharmaceuticals.

Mutagenicity is, in many cases, a precursor of carcinogenicity [3]. A chemical is mutagenic if it causes an increase in the rate of naturally occurring mutations. According to the size of the segment of the deoxyribonucleic acid (DNA) changed, mutations can be on a small scale (i.e., substitution, deletion, insertion of a base pair) or large scale (i.e., deletion, duplication, inversion, translocation of a part of the DNA) [3]. The current lack of mutagenicity data for marketed pharmaceuticals has been acknowledged [13]. Specifically, the study states that out of 838 pharmaceuticals available in the market, 366 (44%) are related to retrievable published mutagenicity or carcinogenicity data, and only 208 (25%) meet all the requirements given in the guidelines for testing pharmaceuticals.

As a result, the evaluation of previously developed and highly marketed pharmaceuticals is considered a stringent necessity. Toward this aim, the present study investigated the mutagenicity of 11 pharmaceuticals, namely, azithromycin, erythromycin, ofloxacin, sulfamethoxazole, trimethoprim, carbamazepine, diclofenac, ibuprofen, metoprolol, metoprolol, and propranolol. The most photolabile pharmaceuticals were further used to assess the effects of their phototransformation products. In addition, a photolysed mixture of diclofenac, ofloxacin, and sulfamethoxazole was evaluated for its mutagenicity and estrogenicity, and its phototransformation products were elucidated by liquid chromatography analysis. Moreover, screening of the gene expression profile was performed as a supplementary endpoint to reveal the mode-of-action of the photolysed mixture of diclofenac, ofloxacin, and sulfamethoxazole in the human genome. Gene expression profiling involves the study of the expression patterns of numerous genes simultaneously and may provide an insight into changes occurring at a molecular level, which represents the initial interaction between environmental contaminants and biological systems [14]. It is useful for unveiling the mode-of-action, which is a first step toward establishment of the adverse outcome pathway.

The methodology designed for the purposes of the present study led to an assessment of the effects of pharmaceuticals in which mixtures of the parent compounds and their phototransformation products were studied concurrently.

### MATERIALS AND METHODS

**Materials**

Atenolol, azithromycin, carbamazepine, diclofenac, erythromycin, ibuprofen, metoprolol, ofloxacin, propranolol, sulfamethoxazole, and trimethoprim (purity > 99%) were purchased from Sigma Chemical. All the reagents used for the assays were at least of analytical grade. A list of all chemicals and reagents is given in the Supplemental Data (Table S1). The terms SC, MC, and 3photMC are used to express the single, mixture, and photolysed mixture of compounds, respectively.

**Experimental design**

Mutagenic effects on L5178Y tk+/- mouse lymphoma cells (CRL-9518, ATCC; Table 1) were evaluated for 1) the 11 pharmaceutical compounds (11SC); 2) a mixture of diclofenac, ofloxacin, and sulfamethoxazole (3MC); and 3) photolysed mixtures of diclofenac, ofloxacin, and sulfamethoxazole (3photMC). It should be noted that higher concentrations (mg/L) than those found in the environment were used for the 11SC and 3MC experiments. The concentrations were selected based on the criteria set in the Organisation for Economic Co-operation and Development guidelines, in which mutagenicity should be tested at concentrations causing high cytotoxicity (10–20% relative total growth for the maximum concentration). Prioritization of the biological potency of the pharmaceuticals was achieved as described in detail in the *Mutagenicity and estrogenicity assays* section. The photolysed mixtures were selected after the development of a central composite design (4 factors, 3 blocks, 30 experiments; Supplemental Data, Table S2). The factors evaluated were the initial concentration of each pharmaceutical and the time of photolysis. Our experimental design provides a way to minimize the number of experiments while providing insights into relationships between the variables [15]. A 2-level factorial design was developed, meaning that all variables were evaluated at 2 levels. The number of experiments was determined from the formula $N = 2k$ (factorial) + $2n$ (axial) + $3 \times 2$ (center), as described

![Table 1. Experimental design](attachment:table1.png)

| Type of mixture$^a$ | AZT | ERY | OFL | SMX | TMP | CMZ | DCF | IBF | ATL | MET | PRP |
|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Single compounds (11SC)$^c$ | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| Mixture (3MC)$^d$ | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| Photolysed mixture (3photMC)$^e$ | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| Mixture (3MC)$^f$ | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| Photolysed mixture (3photMC)$^g$ | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |

$^a$Initial concentration of each compound at the µg/L or mg/L level.

$^b$Pharmaceuticals present in each type of mixture.

$^c$The concentrations were 50 mg/L, 100 mg/L, and 250 mg/L.

$^d$The concentrations were 4 mg/L, 7 mg/L, and 10 mg/L.

$^e$The concentrations were 200 µg/L, 350 µg/L, and 500 µg/L, and photolysis was for 20 min, 40 min, 60 min, or 80 min according to the central composite design (Supplemental Data, Table S2).

$^f$The concentrations were 50 µM.

AZT = azithromycin; ERY = erythromycin; OFL = ofloxacin; SMX = sulfamethoxazole; TMP = trimethoprim; CMZ = carbamazepine; DCF = diclofenac; IBF = ibuprofen; ATL = atenolol; MET = metoprolol; PRP = propranolol.
elsewhere [16]. Toxicity screening of the 30 different mixtures was performed using Daphnia magna, Pseudokirchneriella subcapitata, and Vibrio Fischeri (data not shown). The resulting most toxic mixtures of the 3photMC (7 different mixtures) were further evaluated for their mutagenic and estrogenic effects. Although we were unable to find evidence in the literature that baseline toxicity can relate to any other type of biological effect, we decided to consider toxicity as a criterion for the selection of the mixtures to be tested, as a first step.

**Photolysis of mixtures of pharmaceuticals**

Photolysis of mixtures was performed as described elsewhere [17]. Briefly, the experiments were carried out in a cylindrically shaped reactor irradiated with a UV low-pressure mercury lamp of 9 W (Radium, Puritec, UVC-LPS 9, 100–280 nm, λ = 254 nm). This was done to simulate natural photolysis, because UV is a part of sunlight, and also to provide technological treatment by UV-C. The lamp was immersed in the immersion tube of the reactor, which was made from quartz glass to allow transfer of radiation. The temperature was maintained at 25 °C with a cooling water jacket, and the pH of each solution was adjusted to 7.5 ± 0.1 (using Milli-Q water; Millipore), to simulate actual aquatic conditions. Two samples of 1 mL each were taken at time 0 and at the end of each photolysis experiment, for the bioassays and the chromatographic analysis. The concentrations tested ranged in the μg/L to mg/L range.

**Liquid chromatography analysis**

An ultra-performance liquid chromatography system coupled to an electrospray tandem triple quadrupole mass spectrometer (ACQUITY TQD, Waters) was used for analysis of the samples. The system is combined with an electrospray ionization (ESI) source in positive mode (ES+). Multiple reaction monitoring mode was used for detection and mass analysis in a scan range of 50 m/z to 600 m/z. Samples were injected into a BEH Shield RP18 column (1.7 μm, 2.1 mm × 50 mm; Waters). The method has been described elsewhere [17]. In the framework of the present study, different modes such as full scan, single ion recording mode, and multiple reaction monitoring mode were used for further identification and verification of the possible phototransformation products generated during photolysis. The protonated molecular ion [M+H]+ was selected as the precursor ion for collision-induced dissociation fragmentation to produce MS2 product ion spectra, and then the product ion with the greatest relative abundance was selected for further MSn analysis.

**Mutagenicity and estrogenicity assays**

The mutagenicity tests were performed using the mouse lymphoma assay with the L5178Y tk+/− clone (CRL-9518, ATCC) [18]. This assay is advantageous because it can detect gene mutations (i.e., point mutations or insertion and/or deletion of genes) and to an extent, clastogenicity (i.e., morphological changes in chromosomes). The cells were maintained as a suspension culture in RPMI 1640 medium (sterile-phosphate-buffered saline, twice). The NucleoSpin RNA II kit (Macherey-Nagel) was used for total ribonucleic acid (RNA) extraction of each sample (Supplemental Data, Figure S1). An A260/A280 RNA quality assessment was performed using the GeneChip 3'-IVT Express Kit (Affymetrix) was performed. Gene expression profiling was...
performed using arrays of whole human genome (GeneChip HG U133 Plus 2, Affymetrix). This is 1 of the most comprehensive expression arrays, with information on more than 47,000 transcripts (33,000 well-established genes). The amplified RNA was purified using magnetic beads, heat-fragmented, and hybridized (16 h, 45 °C) onto the microarray, which was thereafter stained and scanned (GeneChip scanner 3000, Affymetrix). An A260 to A280 ratio of 1.9 to 2.1 was considered acceptable. Two microarrays for each experiment were used for the analysis.

Data analysis

The criteria proposed by Honma et al. [19] were used for categorizing colonies and evaluating mutation frequencies. Analysis of variance (ANOVA) was conducted using SPSS software.

A quality control analysis was done to reduce low-quality intensity signals and variation in chip hybridization. Scatter plots were used to create groups for comparisons. Statistical evaluation of the results from the microarrays was done using commercial software (JMP Genomics, Ver 4, SAS Institute), as described elsewhere [20]. In brief, the raw fluorescence intensity values were normalized by applying quantile normalization, robust multiarray average background correction, and median polish probe set summarization. Analysis of variance was used to identify differential messenger (m)RNA expression (SAS Institute). A hierarchical cluster analysis was performed using Ward’s method. The false discovery rate correction method was applied to reduce the false positive rate of 5%. Changes in p values and signal log ratios were calculated. On the basis of these values, statistically differentially expressed genes were identified. Fold changes of \( \geq 2 \), corresponding to a signal log ratio of \( \geq 1 \), with a change in p value of 0.0045 \( \geq p \geq 0.9955 \) for every single comparison were defined as statistically significant. The microarray data have been deposited in the Gene Expression Omnibus of the US National Center for Biotechnology Information [21] and approved, indicating that the standards for minimum information about a microarray experiment were met. They are accessible through Gene Expression Omnibus series accession number GSE77215 (See Data Availability). The gene descriptions provided by the US National Center for Biotechnology Information’s gene database were used to better understand the role of the differentially expressed genes. The given genes were searched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY database as a pathway mapping tool. A statistical enrichment analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery for identifying significant biological processes (gene ontology classification and functional annotation clustering) and the pathways affected, following the protocol of Huand et al. [22].

RESULTS

Proposed transformation pathways and phototransformation products generated during photolysis

The UV-C irradiation of diclofenac, ofloxacin, and sulfamethoxazole was found to generate phototransformation products. This was expected because the emission spectrum of the lamp overlies the absorption spectra of the substances, and hydroxyl radicals through lysis of water molecules were most likely present [23,24]. Low-pressure mercury lamps have 2 emission lines, at 254 nm (the main line) and at 185 nm (the weaker line, 6% of the total intensity).

An attempt was made to elucidate the reaction pathways and mechanisms through the identification of phototransformation products of the 3photMC. Fourteen phototransformation products, characterized by different m/z ratios (Supplemental Data, Figure S2), with peaks greater than 10% of the highest peak intensity are proposed (Figure 1).

As previously reported by Kim and Tanaka [25], acidic drugs such as diclofenac can decompose more easily by direct UV photolysis. Ruptures of the C–Cl and N–H bonds can take place under UV-C irradiation [26]. The phototransformation products proposed in the present study preserve the core structure of diclofenac. Transformation, based on the m/z values and relevant literature, is suggested to occur through a multistep process involving diclofenac derivative hydroxylation reactions through attack of HO•, decarboxylation reactions in the aliphatic chain, and further oxidation steps. Based on the proposed hydroxylation mechanism on the 2 aromatic rings, D1 (m/z 229; RI 30–45%) and D2 (m/z 343; RI 35–65%) were attributed to the formation of dihydroxylated and trihydroxylated photoproducts [27]. Alkylation of the aromatic ring of diclofenac is suggested to occur, producing 2 major analogues of diclofenac, D3 (m/z 215; RI 20–65%) and D4 (m/z 251; RI 15–50%). The elucidation route of D1 can be attributed to the dehydroxylation of the carboxylic group, abstraction of the chloride group at C6 and C2, and further addition of a hydroxyl group to the 2-(2-(2-chloro-6-hydroxyphenyl)amino)phenyl) acetic acid. The intramolecular cyclization proposed in D3 was found to be the predominant direct photochemical reaction of diclofenac. Formation of D3 corresponds to loss of the fragment \(-\text{CH}_2\text{COOH} and the choline moiety of the molecule, followed by a subsequent methylation at the C8 position. Formation of the photoprod D4 may be attributed to alkylation of the aromatic ring at the C8 position and elimination of the \(-\text{CH}_3\text{COO} group.

Phototransformation of ofloxacin is supported by its high molecular extinction coefficient (10970 M\(^{-1}\) cm\(^{-1}\)) at 254 nm [28]. The suggested transformation of ofloxacin relates to the formation of the carboxylic group and the alkyl moiety of an aromatic ring. O1 (348 m/z; RI 20–65%) was found to be 1 of the major phototransformation products, suggested to be formed after demethylation of the ofloxacin piperazinyl ring. O2 (391 m/z; RI 15–30%) may result from oxidation of the N-piperazinyl ring, giving an aldehyde derivative [29]. At all irradiation times O3 (153 m/z; RI 15–35%) was detected. The formation of O3 indicates cleavage of C16–C17, N–C13, and O–C8 as a preferential route, which originated from a series of C–C cleavage products such as the formation of ((E)-2-formyl-1-oxo-3-((3-oxoprop-1-en-2-yl)amino)propan-1-ide. This product eventually leads to low molecular weight compounds, corresponding to aliphatic carboxylic acids and other ring-cleavage compounds. More specifically, O3 is proposed to be formed from the loss of C\(_1\)H\(_4\)FN\(_2\) after removal of an oxygen atom from the structure. O4 (363 m/z; RI 25–55%) may be formed during oxidation of ofloxacin at the piperazinyl substituent, demethylation, and hydroxylation. O5 (378 m/z; RI 25–75%), may be generated after monohydroxylation of the aromatic ring from the nonselectivity of the HO• attack. It should be noted that most of the proposed intermediates (O1, O2, O4, and O5) have structures in which the heterocyclic fluorne ring remains intact during the oxidation process.

Sulfamethoxazole can absorb up to 315 nm and has maximum absorbance at 262 nm [30]. This region overlaps with the maximum radiation of the lamp (254 nm). Breakdown
of the N-H bond and bonds between –SO₂– and its side atoms can take place for all sulfonamides and thus they can easily photolyse [25]. Five of the phototransformation products of 3photMC are attributed to the photolysis of sulfamethoxazole. Attributed to dealkylation of the isoxazole ring of sulfamethoxazole are S₁ (m/z 226; RI 30–60%) and S₂ (m/z 215; RI 15–45%). More specifically, S₁ may result from the opening of the isoxazole ring at the C₄ and C₅ positions and rearrangement of the hydrogen atoms. The elucidation route of S₂ can be attributed to the abstraction of a methyl group at C₁ of the isoxazole structure and further addition of hydrogen atoms to the structure of the m/z 227. The chemical 3-amino-5-methylisoxazole (S₃, m/z 99; RI 40–90%) was attributed to the cleavage between the aminophenylsulfone and methylisoxazoleamine moieties. Cleaved species S₄(A)-(B; m/z 156; RI 25–35%) are also proposed. The cleaved species S₄(A) is assumed to be formed after cleavage of the isoxazole ring at C₁ and loss of the amine moiety of the sulfur atom, and S₄(B) is assumed to be formed after cleavage of the isoxazole ring, removal of the amine group at C₁, and abstraction of the amine group at C₉. The phototransformation product S₅ (m/z RI 192; 15–40%) is proposed to be generated by a combination of the abstraction of –OOH at the sulfur atom, reduction of the amine group at the C₉ of the aromatic ring, and demethylation at position C₄ of sulfamethoxazole.

Mutagenicity and estrogenicity assays

Mutant cells, deficient in thymidine kinase because of the forward mutation in the tk locus (from tk+/− to tk−/−), are resistant to the cytotoxic effect of pyrimidine analogues such as trifluothymidine. Thus mutant cells are able to proliferate in the presence of trifluothymidine, whereas normal cells, which contain TK, are not. The results in terms of the total number of colonies generated for each pharmaceutical are presented in Figure 2.

Compared with the control, atenolol, metoprolol, propranolol, diclofenac, and ofloxacin were mutagenic at the p < 0.001 level. The highest mutagenicity was observed for the β-blockers, with propranolol possessing the greatest mutagenic potential (52.00 ± 6.24). These values were at the same levels as those obtained for the positive control (methyl methanesulfonate; 52.67 ± 2.08). The least mutagenic pharmaceuticals were carbamazepine (8.00 ± 3.46), azithromycin (8.67 ± 6.03), trimethoprim (11.67 ± 2.08), and erythromycin (14.67 ± 3.06). These trends are supported by the mutation frequency and the relative total growth presented in Table 2. The relative survival decreased in the following order azythromycin > carbamazepine > trimethoprim > ibuprofen > diclofenac > erythromycin > sulfamethoxazole > atenolol > propranolol > ofloxacin > metoprolol. When the colonies were categorized by size, it was observed that the most clastogenic was metoprolol, followed by propranolol. At 100 mg/L the mutagenicity of the pharmaceuticals was lower but still at the levels of the positive control. Propranolol (41.67 ± 12.66) was again the most mutagenic, followed by metoprolol (36.00 ± 8.54). In comparison with the control experiment, statistically significant values (p < 0.001) were calculated for atenolol, metoprolol, propranolol, and ofloxacin. The most clastogenic compound at 100 mg/L was found to be metoprolol, followed by propranolol. The relative survival decrease was as
Sulfamethoxazole; TMP

Follows: ofloxacin > atenolol > metoprolol > diclofenac > propranolol. Atenolol, metoprolol, propranolol, and ofloxacin were evaluated at 50 mg/L. Only metoprolol and propranolol were found to be mutagenic at this concentration. Their relative survival followed the order of ofloxacin > propranolol > atenolol > metoprolol. Once more, it was found that the most mutagenic pharmaceuticals belong to the β-blocker family.

Interestingly, the photolyzed mixtures of diclofenac, ofloxacin, and sulfamethoxazole (3photMC) at concentrations of 200 μg/L to 500 μg/L were found to be able to cause mutagenicity at the p < 0.001 level (Figure 3). Sample HHH-20 (19.75 ± 4.42), in which the 3 compounds were added at the highest concentration (500 μg/L) and were photolyzed for the lowest time (20 min) was the most mutagenic, followed by its nonphotolyzed sample HHH-0 (25.50 ± 3.11), MMM-0 (21.00 ± 7.62), MMM-80 (24 ± 2.16), and MMM-40 (20.25 ± 3.86). This finding indicates that the compounds in the mixture acted synergistically. Statistical comparison of the 3MC versus 3photMC samples indicated that only the sample MMM-80 was lower at the p < 0.05 level, demonstrating that irradiation greater than 80 min is needed to deactivate mutagenic components. The clastogenicity followed the pattern of the mutagenicity results. The most clastogenic samples were once more HHH-20, followed by MMM-0, MMM-80, and MMM-40.

None of the photolyzed samples were found to be statistically estrogenic compared to the negative control. Therefore, because no new insight was obtained, the results are presented in the Supplemental Data (Figure S3).

Gene expression profiling

A small variance between the microarray signals was present based on the overlaying Kernel densities (Supplemental Data, Figure S4) and an ANOVA analysis of the response of microarray chips (Supplemental Data, Figure S5). This relates to the fact that a cell line was used and less variability is expected compared with investigation of different cell types. The log2 fold change at the p < 0.05 level was used for the comparisons (Supplemental Data, Figure S6). Out of a total of 17 744 genes, 1326 differed significantly from controls. The number of altered genes (644 vs 216) was higher when cells were exposed to the pharmaceutical mixture (3MC) compared with the photolyzed pharmaceutical mixture (3photMC; Supplemental Data, Figure S6), indicating a lower altered gene expression. The remaining genes (466) were expressed after both types of exposure. Based on hierarchical clustering, 3 clusters were created: 1 containing the controls and 2 containing both the cells exposed to the mixture and the cells exposed to the photolyzed mixture (3MC+3photMC; Supplemental Data, Figure S7). The top 25 differentially up-regulated or down-regulated genes based on the fold change for the cells exposed to 3MC and to 3photMC (Table 3) are shown. As extracted from the websites of the US National Center for Biotechnology Information and GeneCards, the functions of these genes are mostly related to apoptotic mechanisms, the cell cycle, anti-inflammatory responses, tumor prevention, and so on. Half of the genes shown in Table 3, Mixture of diclofenac, sulfamethoxazole, and ofloxacin (i.e., LAMP3, GADD45A, RRAID, MYC, KCNK5, SLC16A9, ZBED8, CASC1, LINC00342, LOXL1-AS1, NTS, SPTSSB) are not present in Table 3, Photolyzed mixture of diclofenac, sulfamethoxazole and ofloxacin. Similarly, half of the genes shown in Table 3, Photolyzed mixture of diclofenac, sulfamethoxazole, and ofloxacin (i.e., DUSP1, ZNF750, INHBE, SNORA72, IER3, SRGN, MIR29C, TSTAZ, LOC100289230, ZBED6, CCDC18, HSPA1B) are not present in Table 3, Mixture of diclofenac, sulfamethoxazole, and ofloxacin. This indicates the differentiated expression profile of the mixture of parent compounds and the mixture of transformation products. Interestingly, the gene ontology of the top-regulated genes of the 3MC (cell cycle, metabolism and/or biosynthesis, and gene expression and/or translation) and the 3photMC (response to oxidative stress, cell death, and apoptosis) experiments differed intrinsically. Based on the KEGG pathway, the top up-regulated genes of the 3MC experiment relate to proinflammatory mitogen-activated protein kinase (MAPK) and the cell cycle pathway, whereas no pathway was identified for the 3photMC top-regulated genes. The down-regulated genes for both 3MC and 3photMC related to acid transport. Antiapoptotic mechanisms were also found to be down-regulated in the 3photMC experiment.

Among the 1326 differentially expressed genes, the groups ABC, CYP, PTG, SLC, TOP, and UGT could be correlated to exposure to the single compounds diclofenac, ofloxacin, and sulfamethoxazole. Based on the gene ontology analysis through
Table 2. Mutagenicity and cytotoxicity of pharmaceuticals

| Pharmaceutical  | Negative control | 50 mg/L PE (%) | 50 mg/L MF (x10^-6) | 50 mg/L RTG (%) | 100 mg/L MF (x10^-6) | 100 mg/L RTG (%) | 250 mg/L MF (x10^-6) | 250 mg/L RTG (%) |
|-----------------|------------------|----------------|---------------------|----------------|----------------------|------------------|----------------------|------------------|
| Antibiotics     |                  |                |                     |                |                      |                  |                      |                  |
| Azithromycin    | Ethanol          | 77             | nd                  | nd             | nd                   | nd               | nd                   | 83               |
| Erythromycin    | ethanol          | 87             | nd                  | nd             | nd                   | nd               | 146                  | 97               |
| Ofloxacin       | H2O              | 120            | 240                 | 68             | 684                  | 60               | 859                  | 36               |
| Sulfamethoxazole| H2O + acetone    | 78             | nd                  | nd             | nd                   | nd               | 402                  | 86               |
| Trimethoprim    | H2O + ethanol    | 112            | nd                  | nd             | nd                   | nd               | 114                  | 99               |
| Anti-inflammator|                  |                |                     |                |                      |                  |                      |                  |
| Carbamazepine   | Ethanol          | 113            | nd                  | nd             | nd                   | 206              | 83                   |                  |
| Anti-inflammatories |            |                |                     |                |                      |                  |                      |                  |
| Diclofenac      | H2O              | 79             | nd                  | nd             | nd                   | nd               | 618                  | 25               |
| Ibuprofen       | H2O + DMSO       | 95             | nd                  | nd             | nd                   | nd               | 579                  | 96               |
| β-blockers      |                  |                |                     |                |                      |                  |                      |                  |
| Atenolol        | Ethanol          | 118            | nd                  | nd             | nd                   | nd               | 751                  | 100              |
| Metoprolol      | H2O              | 154            | 632                 | 75             | 589                  | 54               | 825                  | 28               |
| Propranolol     | H2O              | 118            | 713                 | 62             | 861                  | 38               | 911                  | 16               |
| Positive control|                  |                |                     |                |                      |                  |                      |                  |
| MMS             | H2O              | 98             | 847                 | 79             | 721                  | 96               | 953                  | 88               |

PE = plating efficiency; MF = mutation frequency; RTG = relative total growth; nd = not determined; DMSO = dimethyl sulfoxide; MMS = methyl methane sulfonate.

Database for Annotation, Visualization, and Integrated Discovery, the differentially expressed genes (1326) were related to biological processes (63.4%), molecular functions (58.7%), and/or cellular components (63.6%). In total, 139 biological processes were altered based on the Gene Ontology categories (data not shown). As presented in Table 4, the biological processes with a higher significance of alteration (p value ≥log10 2) were linked to cell death (i.e., regulation of apoptosis, regulation of programmed cell death, and regulation of cell death), cell division (i.e., regulation of cell proliferation and negative regulation of cell proliferation), and cell signaling response to extracellular stimulus (i.e., response to drugs, response to extracellular stimulus, and response to acid). Moreover, the differentially expressed genes were used to identify the most important pathways based on a statistical enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (Table 5). These pathways related to cellular processes (i.e., cell growth and death and transport and catabolism), environmental information processing (i.e., signal transduction), genetic information processing (i.e., transcription and translation), and metabolism (i.e., amino acid metabolism and carbohydrate metabolism). The p53 gene signaling pathway, the MAPK signaling pathway, the alanine, aspartate, and glutamate metabolism pathway, and the spliceosome pathway were identified as the most significantly altered in all exposure scenarios.

**DISCUSSION**

Fourteen phototransformation products are proposed, based on elucidation of the reaction pathways and mechanisms of the mixture of diclofenac, ofloxacin, and sulfamethoxazole. The following competing pathways are suggested: 1) hydroxylation, 2) decarboxylation, 3) methylation, 4) dealkylation and demethylation, 5) cleavage of the bond between the amino-phenylsulfone and the methylisoxazoleamine moieties for sulfamethoxazole, and 6) modifications of the piperazone and quinolone moieties. These mechanisms have been previously reported for the single compounds as well [27,29–35].

One of the main findings of the present study is that mutagenicity of single-pharmaceutical solutions is significantly reduced as the exposure concentration decreases from 250 mg/L to 50 mg/L. Interestingly, a mixture of active pharmaceutical ingredients may act synergistically and produce statistically significant results at concentrations of 500 μg/L. This finding confirms the significance of assessing mixture effects. The findings in the present study for azithromycin, erythromycin, ofloxacin, and diclofenac are in accordance with the findings of previous studies reviewed by Snyder and Green [36]. It should be mentioned that no retrievable mutagenicity data by means of the tk kinase assay were available for sulfamethoxazole, trimethoprim, carbamazepine, ibuprofen, atenolol, metoprolol, or propranolol. The mutagenicity obtained for azithromycin in the present study is in line with that obtained in the studies of Amacher et al. [37], in which a negative result was reported at

![Figure 3. Mutagenicity of mixtures of diclofenac, sulfamethoxazole, and ofloxacin (3MC) and their photolyzed mixtures (3photMC). Error bars represent standard deviation, and statistically significant difference from the nonphotolyzed mixture is marked by asterisks (*, p < 0.05). Concentrations are presented in the following order: diclofenac, sulfamethoxazole, and ofloxacin, followed by the photolysis treatment period. L = 200 μg/L; M = 350 μg/L; H = 500 μg/L.](image-url)
Table 3. Top 25 up-regulated or down-regulated genes based on the fold change

| Symbol          | Entrez ID | Gene name                                      | Summary for gene function | Log fold change | p value (−log10) for 3MC to control |
|-----------------|-----------|-----------------------------------------------|----------------------------|-----------------|-----------------------------------|
| Mixture of diclofenac, sulfamethoxazole, and ofloxacin |            |                                               |                |                              |                                    |
| ANKR30BL        | 554226    | Ankyrin repeat domain 30B-like                | Protein coding related to the cell cycle | 2.20            | 4.60                             |
| GDF15           | 9518      | Growth differentiation factor 15             | Regulating inflammatory and apoptotic responses | 1.98            | 4.12                             |
| CLGN            | 1047      | Calmegin                                      | Endoplasmic reticulum chaperone protein | 1.58            | 4.46                             |
| DDIT3           | 1649      | DNA-damage-inducible transcript 3             | Promotes apoptosis          | 1.40            | 3.33                             |
| HES1            | 3280      | Hairy and enhancer of split 1                | Transcription factor as a response to DNA damage | 1.34            | 2.91                             |
| LAMP3           | 27074     | Lysosomal-associated membrane protein 3      | Protein coding related to adaptive immunity | 1.32            | 3.06                             |
| GDF15           | 9518      | Growth differentiation factor 15             | Regulating inflammatory and apoptotic responses | 1.54            | 3.66                             |
| DDIT3           | 1649      | DNA-damage-inducible transcript 3             | Promotes apoptosis          | 1.34            | 3.25                             |
| HES1            | 3280      | Hairy and enhancer of split 1                | Transcription factor as a response to DNA damage | 1.27            | 2.82                             |
| PCP4            | 5121      | Purkinje cell protein 4                      | Regulates apoptosis         | 1.07            | 2.39                             |
| RRAGD           | 58528     | Ras-related GTP binding D                    | Cell process and signaling pathways | 0.96            | 3.30                             |
| MYC             | 4609      | v-myc myelocytomatosis viral oncogene homolog (avian) | Activation of p38/JNK pathway by p53-dependent and independent mechanisms | 0.94            | 3.57                             |
| KCNK5           | 8645      | Potassium channel, subfamily K, member 5     | K⁺ transport               | 0.94            | 2.58                             |
| SLC16A9         | 220963    | Solute carrier family 16, member 9 (monocarboxylic acid transporter 9) | Protein coding of monocarboxylate transporter | 0.91            | 2.51                             |
| ZBED8           | 63920     | Zinc finger BED-type containing 8             | Cell signaling             | −0.99           | 3.58                             |
| CASC1           | 55259     | Cancer susceptibility candidate 1             | Protein coding related to microtubule binding and beta-tubulin binding | −1.04           | 2.38                             |
| LINC00342       | 150759    | Long intergenic non-protein coding RNA 342    | Non-protein coding RNA related to gene regulation | −1.06           | 2.50                             |
| CDC20B          | 166997    | Cell division cycle 20 homolog B             | Cell division cycle        | −1.06           | 2.74                             |
| LOXL1-AS1       | 100287616 | LOXL1 antisense RNA 1                        | Non-protein coding antisense RNA | −1.07           | 2.09                             |
| SLC6A14         | 11254     | Solute carrier family 6 (amino acid transporter), member 14 | Protein coding of a beta-alanine carrier and may transport both neutral and cationic amino acids | −1.07           | 2.15                             |
| NTS             | 4922      | Neurotensin                                   | Protein coding related to microtubule binding and beta-tubulin binding | −1.12           | 2.85                             |
| ANX1            | 301       | Annexin A1                                    | Anti-inflammatory response and tumor prevention | −1.15           | 4.45                             |
| SPTSSB          | 165679    | Serum palmitoyltransferase small subunit B   | Protein coding that stimulates the activity of serum palmitoyltransferase | −1.23           | 4.48                             |
| DKK1            | 22943     | Dickkopf WNT signaling pathway inhibitor 1   | Related to apoptosis       | −1.24           | 5.04                             |
| MIR205HG        | 642587    | MIR205 host gene                             | Protein coding related to tumor suppression | −1.27           | 2.71                             |
| CFAP126         | 257177    | Cilia and flagella associated protein 126    | Cilium organization        | −1.27           | 4.04                             |
| Photolysed mixture of diclofenac, sulfamethoxazole, and ofloxacin |            |                                               |                  |                              |                                    |
| ANKR30BL        | 554226    | Ankyrin repeat domain 30B-like                | Protein coding related to the cell cycle | 2.44            | 4.78                             |
| GDF15           | 9518      | Growth differentiation factor 15             | Regulating inflammatory and apoptotic responses | 1.54            | 3.66                             |
| DDIT3           | 1649      | DNA-damage-inducible transcript 3             | Promotes apoptosis          | 1.34            | 3.25                             |
| HES1            | 3280      | Hairy and enhancer of split 1                | Transcription factor as a response to DNA damage | 1.27            | 2.82                             |
| CLGN            | 1047      | Calmegin                                      | Endoplasmic reticulum chaperone protein | 1.06            | 3.73                             |
| DUSP1           | 1843      | Dual specificity phosphatase 1               | Stress response and cellular proliferation | 0.94            | 2.87                             |
| ZNF750          | 79755     | Zinc finger protein 750                      | Protein coding related to cell differentiation | 0.90            | 4.14                             |
| INHBE           | 83729     | Inhibin, beta E                              | Cell growth and differentiation | 0.86            | 3.00                             |
| SNORA72         | 26775     | Small nuclear RNA, H/ACA box 72              | Guide site-specific rRNA modification | 0.85            | 3.35                             |
| PCP4            | 5121      | Purkinje cell protein 4                      | Regulates apoptosis         | 0.84            | 2.00                             |
| IER3            | 8870      | Immediate early response 3                   | Protection of cells from tumor necrosis factor (TNF)-alpha-induced apoptosis | 0.83            | 3.56                             |
| SRGN            | 5555      | Sereglin                                     | Inflammatory response and apoptosis | 0.83            | 2.52                             |
| GPMB            | 10457     | Glycoprotein (transmembrane) mmb             | Growth function and metastasis | 0.83            | 3.84                             |
| MIR29C          | 407026    | MicroRNA 29c                                  | Noncoding RNA involved in post-transcriptional regulation of gene expression | −0.79           | 2.00                             |
| SLC6A14         | 11254     | Solute carrier family 6 (amino acid transporter), member 14 | Protein coding of a beta-alanine carrier and may transport both neutral and cationic amino acids | −0.80           | 1.70                             |
| TSTA3           | 7264      | Tissue specific transplantation antigen P35B | Protein coding related to the expression of glycoconjugates | −0.81           | 3.84                             |
| LOC100289230    | 100289230 | Uncharacterized LOC100289230                 | Noncharacterized RNA        | −0.86           | 2.01                             |

(continued)
not genotoxic at a concentration of 100 mg/L. Kadotani et al. [39] and Kuboyama and Fujii [40] found that the mutagenicity of diclofenac was questionable because it was not mutagenic in the Ames test and was slightly mutagenic in the Rec assay using *Bacillus subtilis* cells. Ibufrofen was found to be weakly genotoxic to mice bone marrow cells by means of the sister chromatid exchange and not mutagenic in the Ames test [41]. Robbiano et al. [42] demonstrated that β-blockers are cytotoxic and mutagenic to rat hepatocytes at concentrations comparable with those of the present study. In addition, they reported cytotoxicity for human hepatocytes of N-nitrosodiethylamine derivatives of β-blockers even at lower concentrations (7.8 mg/L). Derivatives of N-nitrosodimethylamine may be created in the stomach after exposure of amine or amide groups to HCl. This finding confirms the importance of evaluating the transformation products of pharmaceuticals. In a study by Okine et al. [43], atenolol, metoprolol, and propranolol were found not to be genotoxic by means of the Ames test and weakly genotoxic by the cytokinesis block micronucleus assay toward HepG2 cells. It can be concluded that a battery of bioassays should always be applied, to be able to assess the existence of different detrimental effects.

Fent et al. [44] evaluated the cytotoxicity of some of the pharmaceuticals evaluated in the present study using the yeast estrogen assay. In their study, atenolol was not cytotoxic, and carbamazepine was cytotoxic at concentrations higher than 236.3 mg L⁻¹ (1 mM). In the case of metoprolol and ibuprofen, these 2 pharmaceuticals had a cytotoxic potential at concentrations above 26.73 mg L⁻¹ and 20.63 mg L⁻¹, respectively (0.1 mM). Propranolol and diclofenac were cytotoxic even at 2.59 mg L⁻¹ and 2.96 mg L⁻¹, respectively (0.01 mM). The same trend was observed in the present study as well.

Nasuhoglu and colleagues [30] indicated that photolysis of sulphonamethoxazole in ultrapure water can result in the generation of transformation products that are more toxic than the parent compound to *D. magna*. Similarly, Jung et al. [45] showed that sulphonamethoxazole and other sulphonamide drugs generate toxic transformation products in *D. magna*. This finding highlights the different sensitivities of the test batteries applied. Some other examples are the studies of Schmitt-Jansen et al. [46] and Schulze et al. [47], which reported an increased phytotoxicity of diclofenac transformation products at concentration levels that may be close to environmental concentrations of residual

concentrations lower than 240 mg/L. Ofloxacin was found to be genotoxic in the SOS Chromotest and mutagenic in the T98 strain of the Ames test at concentrations up to 2.5 mg/L [38]. The same study found sulfamethoxazole to be mutagenic and

**Table 3. Continued**

| Symbol     | Entrez ID | Gene name                                      | Summary for gene function          | Log fold change | p value (−log10) for 3MC to control |
|------------|-----------|------------------------------------------------|------------------------------------|----------------|----------------------------------|
| ZBED6      | 100381270 | Zinc finger, BED-type containing 6             | Transcriptional modulator that affects cell cycle | −0.90          | 2.03                             |
| CFAP126    | 126       | Cilia and flagella associated protein         | Cilium organization                | −0.91          | 3.43                             |
| CDC20B     | 169679    | Cell division cycle 20 homolog B              | Cell division cycle                | −0.92          | 2.49                             |
| ANXA1      | 301       | Annexin A1                                    | Anti-inflammatory response and tumor prevention | −0.93          | 4.05                             |
| DKK1       | 22943     | Dickkopf WNT signaling pathway inhibitor 1    | Relates to apoptosis               | −0.94          | 4.51                             |
| CCDC18     | 343099    | Coiled-coil domain containing 18              | Protein coding related to the cell cycle | −0.94          | 2.22                             |
| HSPA1B     | 3304      | Heat shock 70kDa protein 1B                   | Protein coding protecting against aggregation of proteins | −0.98          | 2.70                             |
| MIR205HG   | 642587    | MIR205 host gene                              | Protein coding related to tumor suppression | −1.05          | 2.38                             |

As extracted from the websites of the US National Center for Biotechnology Information and GeneCards.

Positive values indicate up-regulation and negative values down-regulation.

3MC = mixture of diclofenac, ofloxacin, and sulfamethoxazole; 3photMC = photolysed mixture of diclofenac, ofloxacin, and sulfamethoxazole (15-min irradiation).

**Table 4. Gene ontology of differentially expressed genes**

| Biological process                      | 3MC     | 3photMC  | 3MC+3photMC |
|----------------------------------------|---------|----------|-------------|
| Cell death                             |         |          |             |
| Regulation of apoptosis                | <1      | 2.12     | 1.6         |
| Regulation of programmed cell death    | <1      | 2.06     | 1.54        |
| Regulation of cell death               | <1      | 2.03     | 1.51        |
| Cell division                          |         |          |             |
| Regulation of cell proliferation       | 1.70    | 3.26     | 2.47        |
| Negative regulation of cell proliferation | <1    | 2.62     | 1.44        |
| Cell signaling                         |         |          |             |
| Transforming growth factor beta receptor signaling pathway | 2.10    | 2.43     | 2.09        |
| Metabolism                             |         |          |             |
| Coenzyme metabolic process             | 2.14    | <1       | 1.44        |
| Oxidation reduction                    | 3.04    | 1.24     | 2.33        |
| Response to extracellular stimulus     |         |          |             |
| Response to drug                       | <1      | 2.60     | 1.78        |
| Response to extracellular stimulus     | 1.41    | 2.15     | 1.47        |
| Response to acid                       | <1      | 2.11     | 1.3         |

The major enriched gene ontology terms, followed by a Benjamini test were used to identify altered biological process using the Database for Annotation, Visualization, and Integrated Discovery.

Significantly enriched biological processes compared with control in which a p value (−log10) > 2 are presented.

1110 genes were differentially expressed compared with control and related to 121 biological processes.

882 genes were differentially expressed compared with control and related to 110 biological processes.

1326 genes were differentially expressed compared with control and related to 139 biological processes.

3MC = mixture of diclofenac, ofloxacin and sulfamethoxazole; 3photMC = photolysed of diclofenac, ofloxacin, and sulfamethoxazole (15-min irradiation).
diclofenac after photolysis in distilled water. Lastly, Vasquez et al. [17] reported that during photolytic treatment processes, genotoxic byproducts can be formed that are even more genotoxic than oxytetracycline. The literature regarding estrogenicity of phototransformation products of pharmaceuticals is limited. The aim of most of the research has been to assess the estrogenic potential of parent compounds. For instance, the Yeast Estrogen Assay and the E-screen using MCF-7 cells demonstrated only weak estrogenicity for erythromycin and atenolol and no potential of parent compounds. For instance, the Yeast Estrogen Assay and the E-screen using MCF-7 cells demonstrated only weak estrogenicity for erythromycin and atenolol and no estrogenicity for oxytetracycline and ibuprofen [48]. These findings explain why none of the transformation product samples were found to be statistically estrogenic compared with 17β-estradiol in the present study.

The present study is 1 of the first attempts to evaluate the simultaneous effect of phototransformation products of mixtures of pharmaceuticals. Of utmost importance in the present study was the finding that exposure to the mixture compounds activates expression of different genes, which cannot be accurately predicted when exposure to each parent compound is considered alone. Literature focusing on the effects of pharmaceuticals on gene expression profiles after exposure at environmental levels is scarce. Previous studies have mainly focused on investigating mixtures of parent compounds. Khalaf et al. [49] investigated the response of the nonsteroidal anti-inflammatory drugs diclofenac, ketoprofen, naproxen, ibuprofen, dextropropoxyphene, and trimethoprim using environmentally relevant concentrations. Inflammatory responses (nuclear factor-κB, the MAPK pathway, and activator protein-1) to T24 bladder epithelial cells and Jurkat T-cells were observed. As mentioned in their study [49], both the nuclear factor-κB and MAPK pathways, including activator protein-1, are involved in regulation of cyclooxygenase expression. Nuclear factor-κB and activator protein-1 activation was also observed after exposure to water samples collected from different locations in Sweden. The findings of Khalaf et al. [49] are in accordance with the findings of the present study, because we found that MAPK was also affected after exposure to the mixture of pharmaceuticals. The effects of diclofenac on the MAPK pathway have been thoroughly reported in the literature [50]. In a recent study by Roller et al. [51], diclofenac was found to affect the MAPK pathway in a synergistic manner in the presence of sorafenib, a pharmaceutical used to treat advanced renal cell carcinoma. Genome-wide expression profiling demonstrated synergy-specific down-regulation of survival-related genes. Fluoroquinolones have also been found to affect the MAPK pathway by impairing the function of signaling proteins like β1-integrin, a transmembrane signal receptor, and consequently their essential regulation of cell–cell and cell–matrix interactions [52,53]. Interestingly, sulfamethoxazole was found to regulate cytokine production in primary human differentiated blood macrophages. Sulfamethoxazole reduced lipopolysaccharide-induced MAPK phosphorylation, and consequently caused effects on cytokine down-regulation. Furthermore, sulfamethoxazole inhibited lipopolysaccharide-activated nuclear factor-κB activation. In conclusion, sulfamethoxazole was found to play a role in limiting the propagation of uncontrolled inflammation, via suppression of MAPK and nuclear factor-κB activity, after microbial infections [54].

Differential gene expression in terms of the cell cycle pathway leading to quiescence has been reported previously [55]. Using human embryonic cells, Pomati et al. [55] assessed the gene expression profiles of a mixture of compounds in which oxytetracycline and sulfamethoxazole were present. Their results indicate that the differentially expressed p16 and p21 genes were responsible for the effects on the cell cycle. In the same study, apoptosis was excluded, because p53 was not affected. The p53 protein is known as the genome guardian, because it arrests the cell cycle, enhances DNA repair, and promotes cell senescence or apoptosis. Its activation is induced after stress signals (like DNA lesions, oxidative stress, and so on). Mutations in the p53 gene are correlated with uncontrolled proliferation and tumor development [3]. In the present study, apoptosis is to be expected because the p53 pathway was affected as well. This finding suggests that

| Biological process                  | p53 signaling pathway | Lyosome | MAPK signaling pathway | Wnt signaling pathway | ErbB signaling pathway | Notch signaling pathway | p value (–log10) b |
|-------------------------------------|-----------------------|---------|------------------------|-----------------------|------------------------|------------------------|------------------|
| Cellular processes                  | 1.27                  | 1.13    | 1.70                   | <1                    | 1.51                   | <1                    | 1.27             |
| Transport and catabolism            | <1                    | 1.03    | <1                    | 1.20                  | <1                    | <1                    | <1               |
| Environmental information processing|                       |         |                        |                       |                        |                       |                  |
| Signal transduction                 |                       |         |                        |                       |                        |                       |                  |
| Genetic information processing      |                       |         |                        |                       |                        |                       |                  |
| Spliceosome                         | <1                    | 1.62    | 1.11                   | <1                    | 1.34                   | <1                    |                  |
| Translation                         | <1                    | 1.34    | <1                    | 1.18                  | <1                    | <1                    |                  |
| Metabolism                          |                       |         |                        |                       |                        |                       |                  |
| Amino acid metabolism               | 2.41                  | 1.74    | 2.68                   | 1.41                  | 1.27                   | 1.16                  |                  |
| Cysteine and methionine metabolism  | 1.05                  | 1.60    | 1.82                   | <1                    |                       |                       |                  |
| Carbohydrate metabolism             | 1.16                  | <1      | <1                    |                       |                       |                       |                  |

aAs identified by the Kyoto Encyclopedia of Genes and Genomes pathway.

bDifferentially expressed genes compared with control were used.

3MC = mixture of diclofenac, oxytetracycline, and sulfamethoxazole; 3photMC = photolysed mixture of diclofenac, oxytetracycline, and sulfamethoxazole (15-min irradiation).
diclofenac plays a crucial role in the activation of this pathway, which is in accordance with a previous study on rainbow trout [56]. This suggests that selection of the mixture compounds is pivotal, and criteria are needed to decide which components are to be assessed in future studies [57]. Altered transcription as a result of exposure to the mixture of pharmaceuticals can be implied because the spliceosome pathway has been found to be affected. The spliceosome complex is heterogeneous and consists of proteins and RNA. It plays an essential role in the maturation of precursor mRNA by enabling excision of introns [58]. The role of some of the splicing factors in carcinomas related to drug resistance has been previously reported [60]. The down-regulation of this pathway as a result of exposure to a pharmaceutical mixture that included diclofenac and oxfloxacin has also been reported for *Danio rerio* liver cells [61].

**CONCLUSIONS**

An approach toward the selection of compounds and mixtures of greater environmental and human health concern was applied in the present study. The use of a factorial design helped to decrease the number of experiments and yet provided answers to whether mutagenicity and estrogenicity are to be expected for photolysed mixtures of the selected pharmaceutical compounds. The present study suggests that photolysed mixtures of pharmaceuticals can create similar phototransformation products to those observed when the compounds are photolysed singly. This result indicates that the transformation routes in mixtures are practically the same. However, the use of real matrices in which the photolysed mixtures may occur should be evaluated to account for matrix effects. Mutagenicity of the single compounds tested is not expected at environmental concentrations. A significant finding is that the mixture effect could increase mutagenicity significantly in the μg/L range, underlining the synergistic behavior that may occur. In the present study none of the photolysed samples were found to be statistically estrogenic compared with the negative control. The gene expression profiling we used in the present study was nontargeted and helped to reveal the mode of action of mixtures of compounds, which is a challenging task in environmental risk assessment. From a total of 17 744 genes evaluated, 1326 were differentially expressed, many of which were not related to exposure to the compounds singly. These genes were related mainly to the MAPK and p53 gene pathways. To better understand the effects of the photolysed mixtures, in vivo studies (coupled with reverse transcription polymerase chain reaction) followed by gene expression profiling are proposed for future studies. These studies could target the upstream or downstream molecules that are part of the pathways identified in the present study and could use mutagenicity as the apical endpoint for constructing the adverse outcome pathway for exposure to phototransformation products of pharmaceutical mixtures.

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Data availability—The whole human genome GeneChip HG U133 Plus 2 from Affymetrix was used. The minimum information about a microarray experiment standards were met, and the data were submitted to the GEO database. The GEO submission number is GSE77215 (NCBI tracking system 17751492; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77215).

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