Ozonation products of zidovudine and thymidine in oxidative water treatment

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ABSTRACT

Ozonation is an advanced treatment technology that is increasingly used for the removal of organic micropollutants from wastewater and drinking water. However, reaction of organic compounds with ozone can also result in the formation of toxic transformation products. In the present study, the degradation of the antiviral drug zidovudine during ozonation was investigated. To obtain further insights into the reaction mechanisms and pathways, results of zidovudine were compared with the transformation of the naturally occurring derivative thymidine.

Kinetic experiments were accompanied by elucidation of formed transformation products using lab-scale batch experiments and subsequent liquid chromatography high resolution mass spectrometry (LC-HRMS) analysis. Degradation rate constants for zidovudine with ozone in the presence of t-BuOH as radical scavenger varied between $2.8 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ (pH 7) and $3.2 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ (pH 3).

The structural difference of zidovudine to thymidine is the exchange of the OH-moiety by the azide function at position 3'. In contrast to inorganic azide, no reaction with ozone was observed for the organic bound azide. In total, nine transformation products (TPs) were identified for both zidovudine and thymidine. Their formation can be attributed to the attack of ozone at the C-C-double bond of the pyrimidine-base.

As a result of rearrangements, the primary ozonide decomposed in three pathways forming two different TPs, including hydroperoxide TPs. Rearrangement reactions followed by hydrolysis and subsequent release of H$_2$O$_2$ further revealed a cascade of TPs containing amide moieties. In addition, a formyl amide riboside and a urea riboside were identified as TPs indicating that oxidations of amide groups occur during ozonation processes.

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1. Introduction

Wastewater treatment plants (WWTPs) represent major point sources for a large variety of organic micropollutants (MPs) that are originating from households and industries, due to the incomplete elimination of these compounds during conventional wastewater treatment (Ben et al., 2018; Freeling et al., 2020; Scott et al., 2018). In addition, most organic MPs that are eliminated during wastewater treatment undergo only partial degradation, thus giving rise to the formation of transformation products (TPs). As a result, both parent compounds and their TPs are discharged into receiving rivers and streams by WWTPs (Wang et al., 2018; Weizel et al., 2020; Xu et al., 2017). In order to reduce the emissions of organic MPs by WWTPs, advanced treatment processes are increasingly used (Rodriguez-Chueca et al., 2019; Shin and Lee 2016; von Gunten 2018; Waclawek et al., 2017). Amongst these, ozonation has been shown to reduce the emission of a large variety of organic MPs. Ozone reacts primarily with organic compounds containing electron rich moieties such as C-C-double bonds and amines or aromatic rings (David Yao and Haag 1991; Hermes et al., 2020; Lim et al., 2019; von Gunten 2018). However, the applied ozone doses typically not result in a complete mineralization of the organic MPs but rather to the formation of a multitude of TPs that are discharged into the environment (Bourgin et al., 2018; Hermes et al., 2020; Merel and Zwiener 2016; Zoumpouli et al., 2019).

Antiviral drugs are a class of pharmaceuticals, which are administered during acute (herpes-simplex-virus) and chronic virus infections (e.g. hepatitis-B-virus, human-immuno-deficient-
virus). Most of these compounds are only metabolized partially in the human body and as such are excreted largely unmodified via urine and feces (Acosta et al., 1996; Mosiekiamang et al., 2019; Zong et al., 2007). As a result, a variety of antiviral drugs has been detected in raw wastewater. Highest concentrations of antiviral drugs that are used for treatment of HIV-infections/AIDS have been observed in the Sub-Saharan Africa due to the number of infected individuals (Muriuki et al., 2020; Nannou et al., 2020; Ncube et al., 2018; Scott et al., 2018; Verlicchi and Grillini 2020). Even though antiviral drugs such as zidovudine (ZDV), emtricitabine (EMT), stavudine (SVD) and acyclovir (ACV) can be eliminated by biodegradation in activated sludge treatment. Stable biotransformation products are formed which even have been detected in drinking water (Funke et al., 2016; Prasse et al. 2010, 2011). The presence of antiviral drugs and their TPs in the environment is of concern due to their adverse effects on aquatic wildlife. This includes reduced growth of green algae (Minguez et al., 2016) or toxicity to Daphnia magna (Schlüter-Vorberg et al., 2015). Thus, there is a need to develop technologies that effectively eliminate the parent antiviral natural occurring derivative thymidine (THY).

In this study, the transformation of the antiviral drug ZDV during ozonation of wastewater was investigated. In order to obtain removal of MPs from wastewater (Bourgin et al., 2018) and has also been shown to be effective for the removal of acyclovir and its bio-TP carboxy-acyclovir (Prasse et al., 2012).

In the study, the transformation of the antiviral drug ZDV during ozonation of wastewater was investigated. In order to obtain additional insights into the degradation pathways during ozonation, the reaction mechanisms of ZDV were compared with the naturally occurring derivative thymidine (THY).

ZDV is a THY analogue in which the hydroxyl group in 3'-position of the ribose moiety is replaced by an azide group. ZDV is not completely degraded during conventional wastewater treatment, whereas THY is ubiquitously present as DNA- and RNA-nucleobase in living cells.

2. Materials and methods

2.1. Chemicals and standards

Methanol (LC-grade), formic acid (for MS), ammonium formate (for MS) and thymidine (THY) were purchased from Sigma-Aldrich (Seelze, Germany). Zidovudine (ZDV) and zidovudine-d3 (ZDV-d3) were purchased from Toronto Research Chemicals (Toronto, Canada). Puriﬁcation of ZDV-d3 (for MS) and thymidine (THY) were purchased from Sigma-Aldrich (Seelze, Germany). Zidovudine (ZDV) and zidovudine-d3 were completely degraded during conventional wastewater treatment, whereas THY is ubiquitously present as DNA- and RNA-nucleobase in living cells.

2.2. Ozone stock solution

Ozone was synthesized by an ozone generator (Heyl-Neomeris LAB2B, Hildesheim, Germany) using oxygen as feed gas. The stock solution (~1 mM) was generated by sparging the ozone-oxygen gas into ice-cooled purified water. To determine the concentration of ozone in the solution ozone was measured directly, using an UV photometer at 258 nm with an extinction coefficient $\varepsilon_{300}$ of 3000 M$^{-1}$ cm$^{-1}$ (Prasse et al., 2012).

2.3. Kinetic ozonation experiments

To determine the rate constant of ZDV competition kinetic experiments were conducted at pH 3 and 7. Thymidine was used as competitor for ZDV, because its rate constant is already known ($k_{O3,THY}=3.0 \cdot 10^4$ M$^{-1}$ s$^{-1}$) (Theruvathu et al., 2001). Kinetic experiments were performed as triplicate. Equal concentrations (1.5 μM) of the competitor and target compounds were added to a solution of 50 mM phosphate buffer. In order to exclude the reaction with OH-radicals t-BuOH (1%) was used as radical scavenger. Ozone stock solution was added in under-stochiometric amounts ranging from 0.02 μM to 0.12 μM, using a glass syringe. Samples were taken after ozone was completely consumed (3 h). Subsequently, samples were analyzed using a LC-MS/MS method to determine concentrations of the competitor (C) and the target compounds (T). To calculate the rate constants equation (1) was used, where $[T]$ was the concentration of the target compound, $[C]$ was the concentration of the competitor and $k_{O3,T}$ as well as $k_{O3,C}$ were the rate constants of competitor and target compounds, respectively.

$$\ln \left( \frac{[T]}{[T_0]} \right) = \ln \left( \frac{[C]}{[C_0]} \right) + \frac{k_{O3,T}}{k_{O3,C}}$$

The rate constant $k_{O3,T}$ was determined plotting $\ln([T]/[T_0])$ vs. $\ln([C]/[C_0])$ with $k_{O3,T}/k_{O3,C}$ as the slope of the linear regression, multiplying the slope with the known rate constant $k_{O3,C}$.

2.3.1. LC-MS/MS analysis

Target compounds and competitor were analyzed using an Agilent 1200 series HPLC system (Agilent, Waldbronn, Germany), consisting of a G1367C Autosampler, a G1312B binary pump and a MistraSwitch column oven (Maylab Analytical Instruments GmbH, Vienna, Austria). Chromatographic separation was achieved with a Synergi Hydro RP (4 μm, 150 × 3 mm i.d.) column (Phenomenex, Aschaffenburg, Germany) coupled with a SecurityGuard AQ-C18 (3 mm i.d.) guard column (Phenomenex, Aschaffenburg, Germany). Aliquots of 10 μL of each sample were injected into the LC-MS/MS-system using 0.2% formic acid (A) and methanol + 0.1% formic acid (B) as mobile phases. The flow rate was kept at 450 μL min$^{-1}$ with the following gradient: 0 min 95% A, 3 min 95% A, 14 min 30% A, 16 min 30% A, 16.1 min 95% A, 21 min 95% A.

| Table 1 | Compounds, solvents and concentrations of stock solutions, retention times, MRM transitions (bold printed transitions are the most intense), declustering potential, collision energy, cell exit potential, dwell time, transition ratio and assignment of internal standards (IS). |
|---|---|---|---|---|---|---|---|---|---|---|---|
| compound | stock solutions | LC/MS/MS-parameters | solvent concentration [g L$^{-1}$] | retention time [min] | [M+H]$^+$ | MRM1 | MRM2 | DP | CE1 [eV] | CE2 [eV] | CP1 [V] | CP2 [V] | dwell time [ms] | Assignment of IS |
| THY | H$_2$O | 1.0 | 7.2 | 243 | 127 | 110 | 32 | 17 | 44 | 10 | 10 | 50 | ZDV-d$_3$ |
| ZDV | H$_2$O | 1.0 | 11.2 | 268 | 127 | 110 | 34 | 17 | 45 | 10 | 10 | 50 | ZDV-d$_3$ |
| internal standard | | | | | | | | | | | | | |
| ZDV-d$_3$ | MeOH | 0.1 | 11.1 | 271 | 130 | 113 | 31 | 15 | 43 | 10 | 10 | 50 | |
An API 4000 QTrap (Sciex, Darmstadt, Germany) mass spectrometer operating in the positive electrospray ionization mode (ESI+) was used as detector applying multiple reaction monitoring (MRM). The ESI-parameters were set as follows: collision gas: high; curtain gas: 35 psi; ion source gas 1 and 2: both 45 psi; source temperature: 550 °C; entrance potential: 10 V; ion spray voltage: 5.5 kV.

Two MRM transitions for each analyte and surrogate standard were used for quantification (most intense transition) and qualification, respectively. The compound specific parameters such as declustering potential (DP), collision energy (CE) and the cell exit potential (CXP) were optimized separately for each compound via direct injection in the continuous flow mode. The MRM transitions, their parameters, retention times, dwell times and the transition intensity ratios are listed in Table 1. Concentrations were determined, using a 4 point calibration from 1 μg L⁻¹ up to 1000 μg L⁻¹.

2.4. Ozonation experiments for the identification of transformation products

Batch experiments were performed in 100 mL amber glass bottles, using 50 mM phosphate-buffer (pH 7) and 1% t-BuOH OH-radical scavenger. To facilitate the identification of transformation products, elevated analyte concentrations up to 20 mM were used. The ozone dose dependent degradation was investigated by different analyte–ozone-ratios (1:0, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10). Samples were taken before adding the ozone solution as well as directly after addition and vigorous stirring. Subsequently, samples were measured using the LC-HRMS method.

2.4.1. LC-HRMS analysis

For identification of formed TPs an Accela HPLC-system (Thermo Scientific, Bremen, Germany) coupled with a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) was used. The chromatographic separation was achieved with a Synergi Hydro RP (4 μm, 250 × 3 mm i.d.) column (Phenomenex, Aschaf fenburg, Germany) coupled with a SecurityGuard AQ-C18 (3 mm i.d.) guard column (Phenomenex, Aschaffenburg, Germany). Aliquots of 20 μL of each sample were injected into the LC-HRMS-system. 0.2% formic acid (A) and methanol + 0.1% formic acid (B) were used as mobile phases at a flow rate of 450 μL min⁻¹ with the following gradient: 0 min 95% A, 10 min 95% A, 20 min 30% A, 27 min 30% A, 27.1 min 95% A, 30 min 95% A. The mass spectrometer was used in positive electrospray- ionization-mode (ESI+). Further MSⁿ-experiments were conducted with a mass range of 50–700 m/z at a resolution 60,000. The MSⁿ-spectra were recorded via collision induced dissociation (CID) and higher energy collision dissociation (HCD).

2.5. Ozonation of spiked WWTP effluent

Effluent from conventional WWTP Koblenz (capacity: 320,000 population equivalents, sludge retention time: approx. 12 d, hydraulic retention time: approx. 6 h, total suspended solids: approx. 4.0 gss L⁻¹) was used to conduct spiked ozonation experiments in wastewater. To this end, 100 mL of effluent was spiked with 5 mg L⁻¹ of ZDV before ozone was added in 10-fold molar excess.

3. Results and discussion

3.1. Oxidation kinetics of ZDV

In order to determine the ozone dose required to achieve a complete transformation of analytes, analyte to ozone ratios from 10:1 to 1:10 were tested. As shown in Fig. 1 complete elimination of the analytes was obtained at an analyte:ozone ratio of 1:2. Competition kinetics used to determine rate constants of ZDV revealed a linear correlation with a slope of 1 (Table 2), indicating that ZDV and THY are reacting at the same rate with ozone.

Second order rate constants of ZDV ranged from 2.79 · 10⁴ M⁻¹ s⁻¹ at pH 7 up to 3.18 · 10⁴ M⁻¹ s⁻¹ at pH 3, corresponding to half-lives from 21 to 24 s at concentration levels of 1.5 μM, respectively (Table 2). The absence of a pH dependence is expected considering pKa values of 9.45 for THY, as well as 9.42 for ZDV (Raviolo and Brinon 2011). Similar kinetics in the absence of t-BuOH indicate that contribution of hydroxyl radicals to ZDV and THY degradation is negligible.

3.2. Identification of TP formation

Due to the high affinity of ZDV and THY to sodium ions, most analytes were identified based on detected M+Na-ions instead of M+H-ions. This has been described for THY before (Dubey et al., 2001; Girault et al., 1994).

The stability of M+Na-ions during MS²-experiments required the addition of 0.1% lithium formate to the LC eluents to form M+Li-ions of analytes and TPs which are less stable and thus increases the detection of fragment ions observed in the MS. Identification of transformation products revealed similar products for both ZDV and THY, indicating that both compounds are undergoing similar transformation reactions and pathways (Fig. 2). The primary attack of ozone takes place at the C5—C6 double bond of the thymine base, leading to the primary ozonide intermediate (IM) E1 according to the Criegee mechanism, confirming the previously described THY reaction mechanism with ozone (Flyunt et al., 2002; Girault et al., 1994).
Homolytic cleavage of IM1 followed by rearrangement results in the formation of TP2. MS²-experiments of TP2 exhibited a main fragment [146.0328 Da], which will be further discussed below for TP4. Subsequent loss of CO which has previously been postulated by Cadet et al. (1999) then results in the formation of TP3. MS²-experiments of TP3 exhibited the same main fragment [146.0328 Da] as TP2. TP4 [146.0328 Da] is formed by oxidation and subsequent cleavage of the "ribose"-nucleobase bond of TP3. MS²-experiments showed the cleavage of H₂O₂, indicating that TP4 should be a cyclic hydantoin hydroperoxide (SI-Table 1) (Flyunt et al., 2002). Furthermore, oxidation of TP3 presumably leads to formation of TP9 via an intramolecular oxidation of the amide carbonyl function, resulting in most likely IM4, an 1,2-dioxetane (Shimomura 2005). Subsequently, cleavage of the O–O- and C–C-bonds results in an unstable carbamic acid and acetyl derived amine. IM5 releases CO₂ forming TP9, revealing the instability of dioxetanes under standard conditions (Vacher et al., 2018). Furthermore, the formation of additional TPs indicated that reaction of ozone with ZDV and THY also result in heterolytic cleavage of the ozonide intermediates (IM2,3), followed by hydrolysis and/or cleavage of hydrogen peroxide (IM6,7) leading to the pyruvic derivate TP5. The most intense fragment observed in MS²-experiments of TP5 showed the loss of pyruvic amide (SI-Table 2).

On one hand a formal loss of carbon monoxide then leads to TP6, which could also be formed by cleavage of the hydroperoxide TP3. The cyclic hydantoin-derivative and the linear urea-derivative
are tautomeric to each other. Although, published results suggested that the equilibrium should be on the side of the cyclic hydantoin-derivative, hemiaminals tend to cleave into its linear tautomer (Iwasawa et al., 2007). Hence, results did not allow for identifying the predominant tautomer.

On the other hand cleavage of methylglyoxal from TP 5 revealed the formation of the formyl urea derivative TP 8. The loss of the formyl-group of TP 8 led to TP 7, whereas TP 10 is formed from the loss of formyl amide.

In comparison to results from literature (Flyunt et al., 2002; Girault et al., 1994), our analytical approach using a direct injection HRMS method allowed to experimentally confirming the formation of TP 2, TP 4 and TP 5 which have not been identified as ozonation products of THY before. The proposed pathway for THY is in accordance with TPs identified and suggested by Girault et al. (1994), Cadet et al. (1999) and Flyunt et al. (2002) and synthesizes the current knowledge about the different reaction mechanisms. The obtained results demonstrate that the pathway proposed for THY can be transferred directly to ZDV, indicating that the azide moiety has no significant impact on the degradation mechanism.

It should be noted that, the reactions of ZDV and THY with other oxidants such as permanganate or hydroxyl radicals lead to similar TPs (Kulkarni et al., 2016; Theruvathu et al., 2001; Wagner et al., 1994).

Ozonation experiments in WWTP effluent with spiked ZDV (5 mg L⁻¹) revealed the formation of the same TPs as observed in buffered ultrapure water with highest peak areas obtained for TP 7, 8, 10 (Fig. 3). Temporal trends of TPs from ozonation of ZDV and THY revealed that all identified TPs are present in the first sampling point (after 30 s stirring), as shown in SI-Figs. 1 and 2. Within eight hours most of the TPs showed decreasing peak areas (PA), most likely resulting from their continuous degradation in solution.

In addition to reactions with ozone, most described reactions could also be attributed to hydrolysis. For example, hydantoins and imides have hydrolysis half-lives of hours, e.g. shown for the pesticides iprodione and flumioxazine (Authority 2014, 2016). Organic-bound azides will not be degraded using ozonation, emitting a slightly mutagenic potential into the environment (Matsumura et al., 1995).

It is remarkable that organic hydroperoxides were scarcely identified with direct injection LC-HRMS methods (Ito et al. 2015, 2020). Furthermore, ozonation transformation processes could result in the same products as naturally occurring oxidation reactions in the human body, as the urea-TP 7a and formamide-TP 10a of THY were found as potential lesion of DNA/RNA resulting from ROS reactions (Cadet et al., 2012; Irvos et al., 2014; Toga et al., 2009).

4. Conclusion

- Ozonation of ZDV and THY followed the same transformation pathway starting with the ozone attack at the C–C-double bond. Based on this activation, ZDV and THY formed nine equivalent TPs each. All transformation reactions took place at the nucleobase, the “riboside”-moiety was not attacked. The azide-moiety of ZDV and hydroxyl-moiety of THY remained inert and had no major impact on the reaction mechanisms.
- Three of the nine TPs were identified as hydroperoxides, remarkably the HRMS-method was able to measure these reactive compounds via direct injection, as fragmentation experiments showed the loss of H₂O₂. The proposed pathway led to an acetamide-TP, a formamide-TP and a urea-TP as endpoints.
- Based on newly identified and previously reported TPs, a comprehensive mechanistic pathway for the reaction of thymidine with ozone was obtained.
- All reactions of the transformation pathway could also be attributed to (a)biotic hydrolysis reactions, thus ozonation was capable of activating compounds being stable in biological treatments.
- The present study revealed the necessity to identify transformation products of antiviral drugs formed during ozonation. An in-depth understanding of oxidation reactions can lead to a better evaluation of potential effects on the environment.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wroa.2021.100090.

References

Acosta, E.P., Page, L.M., Fletcher, C.V., 1996. Clinical pharmacokinetics of zidovudine: An update. Clin. Pharmacokinet. 30 (4), 251–262. Authority, E.F.S., 2014. Conclusion on the peer review of the pesticide risk assessment of the active substance flumioxazine. EFSA J. 12 (6), 3736. Authority, E.F.S., 2016. Peer review of the pesticide risk assessment of the active substance iprodione. EFSA J. 14 (11), e04605. Ben, W., Zhu, B., Yuan, X., Zhang, Y., Yang, M., Qiang, Z., 2018. Occurrence, removal and risk of organic micropollutants in wastewater treatment plants across China: comparison of wastewater treatment processes. Water Res. 130, 38–46. Bourgin, M., Beck, B., Boehler, M., Borowska, E., Fleiner, J., Salti, E., Teschner, R., von Gunten, U., Siegrist, H., Mc Ardell, C.S., 2018. Evaluation of a full-scale wastewater treatment plant upgraded with ozonation and biological post-treatments: abatement of micropollutants, formation of transformation products and oxidation by-products. Water Res. 129, 485–498. Cadet, J., Delatour, T., Douki, T., GASPARUTTO, D., Pouget, J.P., Ravanan, J.L., Sauvaigo, S., 1999. Hydroxyl radicals and DNA base damage. Mutat. Res. Fund Mol. Mech. Mutagen 424 (1–2), 9–21. Cadet, J., Ravanan, J.L., TavernaPuro, M., Menoni, H., Angelov, D., 2012. Oxidatively
