# Impact of Degradation Products of Sulfamethoxazole on Mammalian Cultured Cells

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**ABSTRACT:** Sulfamethoxazole (SMX) is a widely used antibiotic which has been detected in surface water samples in the ng/L range and also detected in drinking water samples. To limit the environmental impact, ozonation treatment of waste streams has been proposed. However, the degradation products created by ozonation as well as their toxicity have not been reported. In this study, we investigated the degradation products of SMX formed during ozonation and the effects of these products on mammalian cultured cells. In addition to alcohols and nitrates, sulfanilamide was identified as the larger molecular weight compound of the degradation products detected. Cells exposed to the degradation products of SMX maintained their polyhedral geometry longer than the control cells. Proliferation of the cells exposed to the degradation products was not negatively affected when compared with the control cells. The results of this study show that bioactive degradation products can be formed by ozonation of SMX.

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**Keywords:** antibiotics; degradation products; mammalian cells; ozonation; sulfamethoxazole

## INTRODUCTION

Antibiotics are widely used on humans and animals in order to treat various bacterial infections. In the United States, around 16,200 tons were produced in 2000 (Kummerer, 2003), and the annual consumption of antibiotics worldwide has been estimated at 100,000–200,000 tons (Lindberg et al., 2004). These antibiotics, as other pharmaceuticals used on organisms, end up in the environment via treated and untreated excreta. Up to 90% of an administrated dose may be excreted unchanged via urine or feces (Al-Ahmad et al., 1999; Daughton and Ternes, 1999; Hirsch et al., 1999). Disposal of unused pharmaceuticals and by-products is another important source of pharmaceutical pollution as well as runoff from manure which can reach groundwater and potentially drinking water (Kummerer, 2003). The presence of antibiotics in the environment is of great concern since it can alter ecosystems and favors the development of resistant bacteria (Boreen et al., 2004).

Municipalities are currently relying on conventional wastewater treatment to remove pharmaceuticals from water streams. However, these systems are not designed to treat pharmaceutical waste and therefore they are released in the environment. Antibiotics have been reported in the effluent from sewage treatment plants (STPs) in Germany (Hirsch et al., 1999), Greece, Italy, France, and Sweden (Andreozzi et al., 2003) and Canada (Metcalfe et al., 2003). Antibiotics have also been detected in surface waters in many countries such as Switzerland, Germany, and the United States in concentration in the low $\mu$g/L (Heberer, 2002). For example, sulfonamides have been repeatedly

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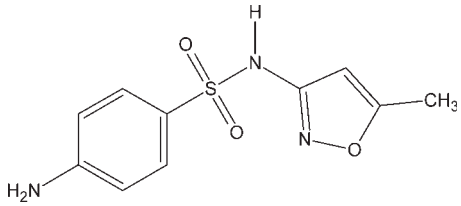

**Fig. 1.** Structure of SMX.

found in environmental samples collected in the United States at concentrations in the 0.13–1.9 $\mu$g/L range (Boreen et al., 2004).

Sulfamethoxazole (SMX) is the most prescribed antibiotic in the United States. SMX is a bacteriostatic antibiotic which interferes with the production of dihydrofolate often prescribed along with trimethoprim (TMP), an antibiotic that binds to bacterial dihydrofolate reductase. Although both antibiotics are bacteriostatic, the combination of TMP and SMX has a bactericidal effect. SMX is used to treat genitourinary tract infections, venereal infections (uncomplicated gonorrhea and chlamydia), gastrointestinal infections, respiratory infections, and central nervous system infections (Kielhofner, 2005). SMX is also used in veterinary practice, aquacultures, and in agriculture as an herbicide (Boreen et al., 2004). SMX has been detected repeatedly in surface water at concentrations of 70–150 ng/L, and in secondary wastewater effluent at concentrations of 200–2000 ng/L (Dodd and Huang, 2004). The molecular structure of SMX is given in Figure 1.

The degradation of SMX by ozonation has been reported as a promising method for the mitigation of risks associated with its presence in the environment (Ikehata et al., 2006). Although ozonation is a well-known process, little is known about the degradation of pharmaceuticals by ozonation. Moreover, the formation of degradation products as well as the toxicity of these products has yet to be determined. In this study, we identify the degradation products of SMX formed during ozonation and assess their toxicity on mammalian cells.

## MATERIALS AND METHODS

### Material

SMX (Sigma-Aldrich, CAS # 723-46-6, purity of 98%) was dissolved in deionized water and the pH was adjusted to 8.0 with phosphate buffer solution. Two different concentrations were prepared for ozonation, 80 mg/L and 200 mg/L, for the identification of the degradation products and the study of the impact of degradation products on mammalian cells, respectively. Although the concentrations used are not environmentally relevant, they facilitated the identification of SMX degradation products and the study of their impact on cell viability.

### Ozonation Experiments

Ozonation experiments were performed using a 2-L semi-batch reactor. An ozone generator OZOMAX model OZO4VTT fed with oxygen was used to produce the $O_3/O_2$ mixture introduced into the solution through a diffusion plate made of porous stainless steel (Mott Corporation, 2 $\mu$m), installed at the bottom of the reactor. The 2-L reactor was made of acrylic in order to perform visual observations during the degradation experiments and to resist $O_3$. The average ozone concentration determined by the Indigo method (Standard method for the examination of water and wastewater, 4500-$O_3$ Ozone-Residual) was 5 mg/L. After 4 min of ozonation, samples were collected using a manifold system (Supelco, Visiprep$^{TM}$) and a vacuum pump.

Upon collection of the samples, sodium sulfite was added with a ratio of 0.02 mL per mL of sample to quench the remaining ozone. Prior to the treatment of cultured cells, the pH of the samples was adjusted to 7.2 using sodium hydroxide.

### Detection of SMX and Its Degradation Products

Concentrations of SMX were monitored by high-performance liquid chromatography (HPLC, Agilent 1100) equipped with a variable wavelength detector. Eluents consisted of 20 mM $NaH_2PO_4$ and acetonitrile using an eluent gradient from 10% acetonitrile to 60% over 15 min (Zorbax SB-C18 column and guard cartridge SB-C18). Samples collected were filtered using 13 mm syringe filter (PVDF, 0.22 $\mu$m, Fisherbrand) and stored in 2 mL-amber bottles at 4°C.

Mass spectrometry coupled to gas chromatography (GC-MS) was used to identify some of the degradation products. All experiments were performed on a Thermo (Trace GC 2000) gas chromatograph equipped with a RTX-5MS column and a Thermo (GCQ/Polaris) mass spectrometer. Helium was used as the carrier gas. The software Excalibur, version 1.1, was used to interpret results obtained through scan mode.

To further investigate the degradation products formed during the ozonation, samples were analyzed by Fourier Transformed Infrared Spectrometry (FTIR). The aqueous samples collected were evaporated at 60°C to dryness with a nitrogen flow. The residue was then placed on a single bounce UATR (Universal Attenuated Total Reflectance) Accessory. A mid range scan was then performed using a Brucker FTIR.

On the basis of the chemical structure of SMX, some assumptions were made regarding potential degradation products formed during the ozonation process. Expected

degradation products were methanol, ethanol, and phenol. Gas chromatography was used to confirm the presence of those compounds in a quenched solution. Analyses were performed on an Agilent 5890 gas chromatograph equipped with a Stabilwax column and using Helium (He) as carrier gas. Using an ion chromatography method, the degraded samples were also tested for nitrites and nitrates. A Dionex DX-100 ion chromatograph was used to performed analyses with an AS14 column and an AG14 guard column.

## Mammalian Cell Treatment

The mammalian cell line used for experimentation was human hepatocellular carcinoma cells or HepG2 cells (ATCC #CRL-11997). The human primary liver cancer cells were chosen due to their long proliferation time. HepG2 cells were grown in T-75 flasks with Eagle's minimum essential medium that was supplemented with fetal bovine serum (5% v/v), and penicillin–streptomycin (1% v/v). Cells were kept in an incubator at 37°C with 5% $CO_2$.

Once the HepG2 cells were confluent, they were seeded into Petri dishes and 96-well plates (both treated for cell culture) at a cell density of $1 \times 10^5$ cells/mL. Cells were incubated and left to adhere to the new surface for 24 h. Following this 24 h period, cells were treated with a given volume of either a solution containing the SMX degradation products, the ozonated water, a phosphate-buffered saline (PBS) solution, or fresh media. Cells treated with PBS, ozonated water, and fresh media were used as control cells to consider the cellular effects because of media replacement. To study the influence of the degradation products concentration on the cells, various concentrations of degradation products were used. The solution containing the products was mixed with cell media based on different dilution factors (volume cell media/volume degradation products solution) of 40, 20, 10, and 5. These dilution factors were chosen based on previous research and literature (Laville et al., 2004). PBS and ozonated water control cells were treated using the same ratios of cell media to control solution. It is also important to note that the same amount of sodium sulfite that was added to the ozonated solution was also added to the control solutions to obtain comparable results. Once treated, cells were incubated for the designated amount of time, either 2, 4, 6, or 8 days at which point observations were taken, and various assays described in the next section were performed to evaluate the impact of SMX degradation products on mammalian cultured cells.

For each time point, cells that were seeded and treated in Petri dishes were used for visual observations. Images were captured using a digital camera (Leica DC) attached to a microscope (Leica DMIL) to identify any morphological changes.

The number of cells in each Petri dish at each time point was measured using a cell and particle counter (Z2 Coulter Counter, Beckman Coulter, On). In each case, the floating and attached cells were counted separately and the ratio calculated to estimate the number of viable and dead cells.

To evaluate the metabolic activity of the treated and control cells, an MTT proliferation assay (Invitrogen, On) was performed on the cells seeded in the 96-well plates at every time point (2, 4, 6, and 8 days) for all dilution factors (media/solution) studied. The color change created by metabolically active cells was measured spectrophotometrically at a wavelength of 570 nm (Benchmark Plus microplate spectrophotometer, Biorad). A series of 96-well plates with no cells, containing the treatment solutions only (degradation products solution, $N = 18$; PBS solution, $N = 18$; ozonated water, $N = 12$; and media, $N = 9$) at all concentrations levels and time points were also analyzed with the plate reader. These background optical densities were averaged and subtracted from the optical densities obtained from experiments using the HepG2 cells. The resulting optical density was therefore due only to the metabolic activity of the cells.

# RESULTS

## Identification of Degradation Products

An important degradation product formed during the ozonation of SMX was identified by GC-MS. This compound, shown on Figure 2, is known as sulfanilamide. GC-MS analysis of the initial SMX solution confirmed that sulfanilamide is not initially present in the solution. GC-MS analysis of the stock solution after 2 weeks of storage at room temperature also indicated that sulfanilamide is not a natural degradation product of SMX. Quantitative analysis showed that sulfanilamide accounts for 2% of the initial mass of SMX. This compound is an antibiotic and it has been used in as a treatment for "coccus" infections. Sulfanilamide was one of the first successfully used antibiotics and is the grandparent of the sulfonamide family of drugs that are commonly used nowadays.

The FTIR spectra indicated intensities of absorbance at frequencies of 1715, 1362, 1222, 1090 $cm^{-1}$ and supported the identification of some structures that might be present in the aqueous solution after the ozonation process. Carbonyl groups (1715 $cm^{-1}$), nitrites (1362 $cm^{-1}$), sulfoxide groups (1222 $cm^{-1}$), and alcohols (1090 $cm^{-1}$) were

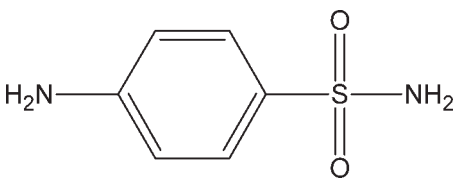


**Fig. 2.** Chemical structure of sulfanilamide.

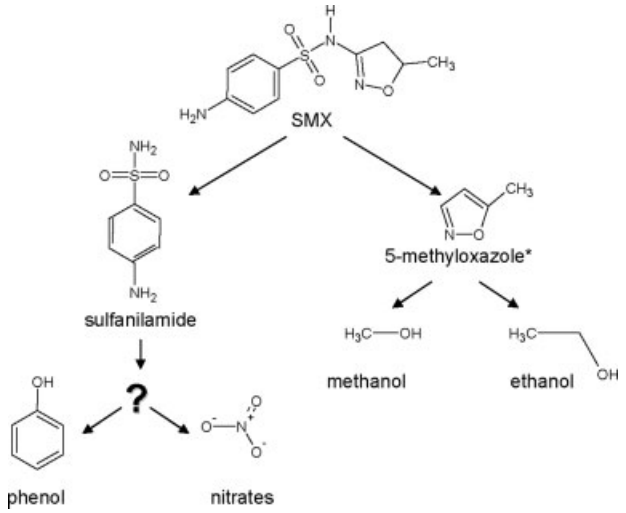


**Fig. 3.** Partial SMX degradation mechanism proposed (*compound not detected).

identified by the FTIR analyses as potential candidates. The presence of nitrites was supported by visual observations; during the ozonation, the color of the solution changed from transparent to slightly yellow. Other groups identified are also consistent with the structure of SMX presented on Figure 1.

Assumptions made regarding the formation of methanol, ethanol, and phenol were validated by gas chromatography and supported by the FTIR results. Analyses performed with an ion chromatograph confirmed the presence of nitrates in solution treated by ozonation while nitrites were not detected. These analyses provided invaluable information on the degradation products formed, as shown on Figure 3, but intermediate products have yet to be identified in order to elucidate the degradation mechanism of SMX during ozonation.

## Effect on HepG2 Cells

As exposure time progressed there was a noticeable difference in the morphology of the SMX degradation product treated cells as compared to the media control cells and PBS treated cells. Surprisingly, the SMX degradation product treated cells were as healthy as or better than the control cells especially at high concentrations. Cloudy cell clumps were observed in PBS treated cells at the 6 and 8 day time points. Figure 4 shows the phase contrast microscopy images of the cells at 8 days. In the media control cells and PBS treated cells [Fig. 4(A,B)], noticeable blebbing and shrinking occurs. In the cells treated with high concentrations of SMX degradation products [Fig. 4(C,D)], cell

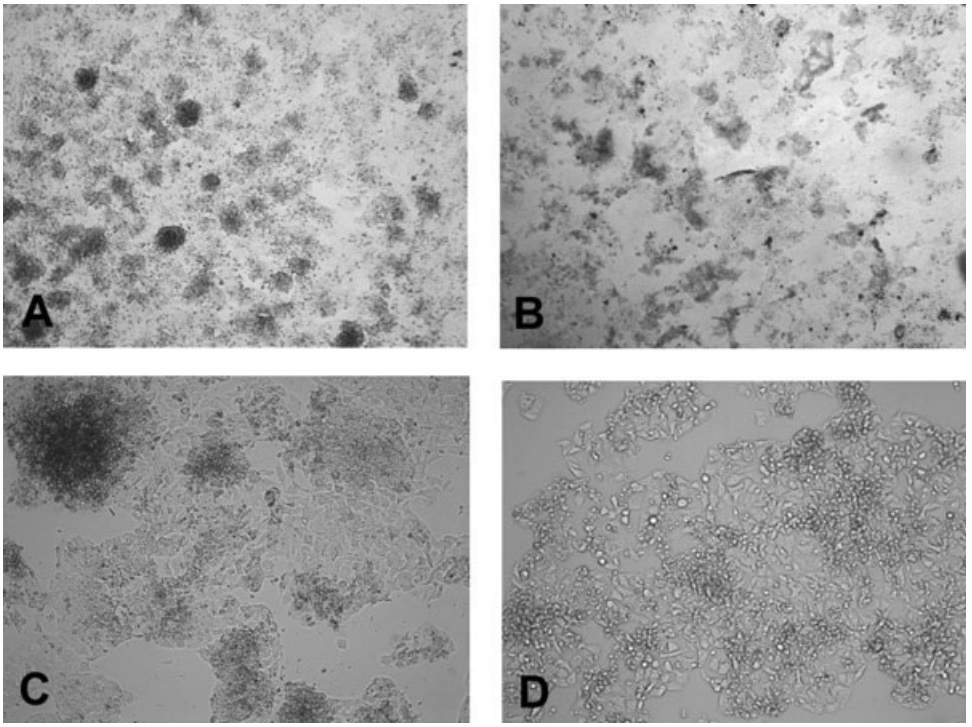


**Fig. 4.** Light microscope images of HepG2 cells 8 days after treatment. (A) Media only, (B) media with the equivalent volume concentration of PBS, (C) degradation products of SMX with a dilution factor of 10, and (D) degradation products of SMX with a dilution factor of 5.

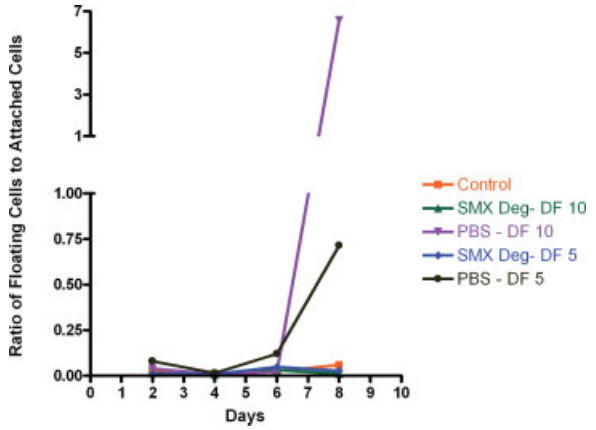


**Fig. 5.** Ratio of floating cells to attached cells for selected dilution factors of SMX degradation products and controls. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

colonies were still evident and the cells still displayed a polyhedral geometry.

Cell counting confirmed that PBS treatment at dilution factors of 10 and 5 had a significant negative effect on the ratio of floating to attached cells at 8 days, Figure 5.

The metabolic activity of the treated and control cells were evaluated using the MTT proliferation assay. Exposure to the parent compound (SMX) did not produce any effect on cell viability (data not shown). Figure 6 shows the absorbance of cells treated with the SMX degradation products solution or the equivalent PBS or ozonated water volume replacement at varying dilution factors and time points. Time ($P < 0.0001$, two-way ANOVA) but not concentration had a significant effect on the proliferation of cells incubated with SMX degradation products [Fig. 6(a)]. For the PBS treated cells, both time and concentration had a significant effect ($P < 0.0001$, two-way ANOVA) on the viability of the cells [Fig. 6(b)]. In the ozonated distilled water treated cells, time had a significant effect ($P < 0.0001$, two-way ANOVA) but not concentration. However, there was significant interaction between the time and concentration ($P = 0.032$) making it difficult to interpret the $P$ values of these results [Fig. 6(c)].

Figure 7 shows the comparison of the proliferation over time for the cells incubated with SMX degradation products, PBS, ozonated water, and control cells exposed only to media. At low dilution factors, the metabolic activity is highest in the SMX degradation products treated cells. A one-way ANOVA was conducted for each concentration (and control) to identify differences in proliferation rates. Table I summarizes the post-test (Bonferroni's Multiple Comparison Test) significance. A difference in cell viability was evident between the SMX degradation treated cells and PBS treated cells. There were significantly less viable PBS treated cells than SMX degradation product treated cells for all dilution factors. At high dilution, this significance is only seen at 2 days, however, with increased concentration, the difference persists. After 4 days, high concentrations of degradation products of SMX produced more viable cells than cells incubated in cell culture media alone.

## DISCUSSION

The degradation of SMX by ozonation has been reported as a promising method for the mitigation of risks associated with its impact on the environment. Under the experimental conditions we used for the ozonation experiments, degradation efficiencies higher than 99% were observed, which confirm the efficiency of ozonation in degrading SMX. We,

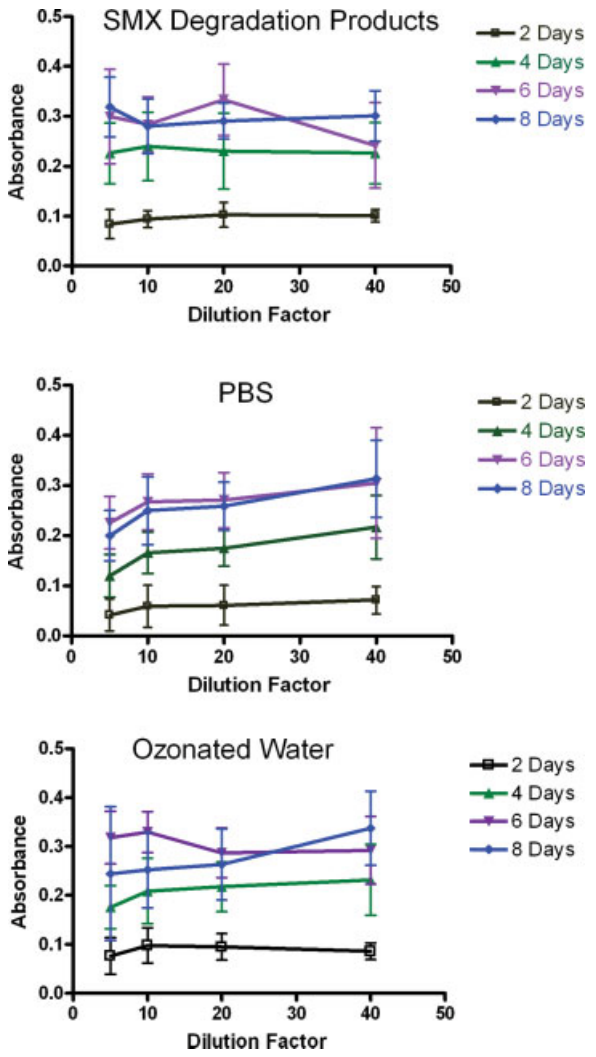


**Fig. 6.** MTT proliferation assay absorbance for the treated cells (bars represent standard deviation). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

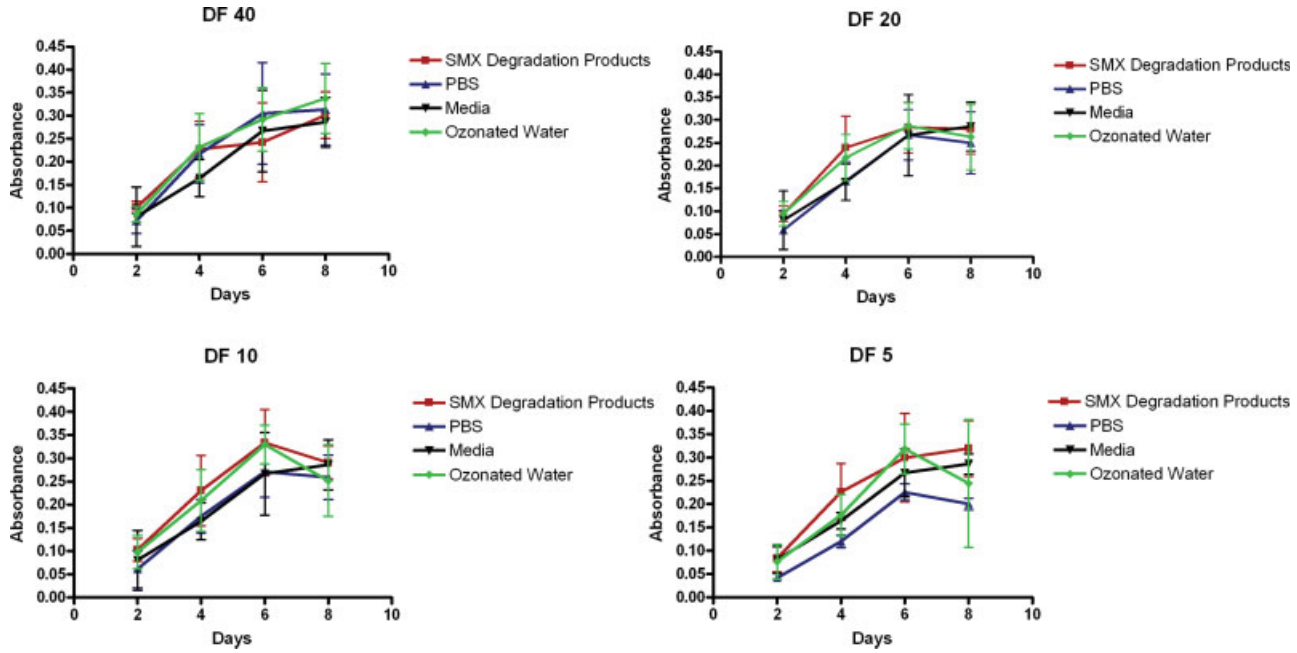


**Fig. 7.** MTT absorbance at each time point for the various test conditions (bars represent the standard deviation). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

however, showed that among other degradation products, sulfanilamide, an active sulfonamide, was produced during the ozonation of solutions containing SMX and accounts for 2% of the initial mass of SMX. We also showed that the mixture of degradation products produced during ozonation have an impact on HepG2 cells.

Sulfanilamide is the active agent of Prontosil, the first sulfa drug discovered and from which more than 15,000 sulfomanide derivatives have been produced. Ozonation caused cleavage at the sulfonamide R-group resulting in the production of a different sulfonamide antibiotic. The production of sulfanilamide would not mitigate the environmental impact of SMX considering that the abatement of the antimicrobial activity would not be achieved. The risks of creating bacteria resistant to sulfonamide would not be mitigated by an ozonation treatment performed under the conditions studied.

Sulfonamides based drugs inhibit folate synthesis in bacterial cells, therefore limiting growth. In mammals, folate is acquired from diet and therefore sulfonamides are not known to have any major toxic effect. We showed that exposure of HepG2 cells to media mixed with the degradation products of SMX does not deter cell proliferation. In fact, proliferation rates were highest in the SMX treated cells when compared with cells exposed to straight media and a control mixture of dilutent (PBS) mixed with media (Figs. 6 and 7). The dilution factor of PBS had a significant effect on cell proliferation [Fig. 6(b)], where as ozonated SMX and ozonated water had no discernable concentration effect [Fig. 6(a,c)]. Surprisingly, there was a noticeable positive effect on the cells treated with SMX degradation products. At the highest degradation products concentrations (dilution factors of 10 and 5), a difference in morphology was evident at late time points along with a significant increase in cell metabolism when compared with cells exposed to media only. These cells retained a normal polyhedral morphology (Fig. 4), whereas the control cells and cells treated with ozonated water and PBS had begun to bleb and shrink. Sulfanilamide is known to be metabolized by parenchymal

**TABLE I. One way ANOVA post-test significance found between treatment types for each concentration and time point**

| Dilution factor | 2 Days | 4 Days | 8 Days |
|---|---|---|---|
| 40 | *SMX > PBS | | |
| 20 | *SMX > PBS | *SMX > PBS | |
| 10 | *SMX > PBS | **SMX > PBS | |
| | | **SMX > Control | |
| 5 | **SMX > PBS | ***SMX > PBS | ***SMX > PBS |
| | | *SMX > Control | *SMX > OW |
| | | | *Control > PBS |

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

liver cells by acetylation (Morland and Olsen, 1977; Suolinna, 1980). The effect of the ozonated products on the cells at late time points could be due to any of the degradation products.

It can be concluded that treating HepG2 cells with a SMX degradation products solution creates a noticeable change in the morphology of the cells; further investigation is necessary to determine what cell functions are affected by these morphological changes. Results from the MTT assay allow us to conclude that the degradation products solution does not negatively effect the proliferation of the HepG2 cells.

The assistance of Christine Leclair for the ozonation experiments are also gratefully acknowledged.

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