Several pharmaceuticals impaired harbor seal lymphocytes (*Phoca vitulina*) *in vitro*

C. Kleinert,¹ M. Mournier,^{1,2}
M. Fortier,¹ P. Brousseau,¹
S. DeGuise,³ M. Fournier¹

¹Institut National de la Recherche Scientifique (INRS)-Institut Armand-Frappier, Laval, Canada; ²UFR des Sciences Pharmaceutiques et Biologiques, Université de Montpellier, France;

³Department of Pathology, University of Connecticut, Storrs, CT, USA

Introduction

In human and veterinary therapeutics, approximately 3000 different drugs are used and prescribed in high quantities.¹ The main substance classes are steroidal hormones, antimicrobials and pharmaceuticals and their metabolites.^{1,2} These compounds could become problematic in the environment, because >80% of the drugs pass the body without being biotransformed, and furthermore their elimination in sewage treatment plants is incomplete.^{1,3} Additionally, the compounds are constructed for a specific intended effect on living beings, and therefore show intrinsic physico-chemical behavior.⁴ Since the compounds are chemically different and peer-reviewed ecotoxicological data is available for less than 1% of human pharmaceuticals, the effects on most trophic levels of aquatic life are hard to predict for a single substance or a mix of various ones.⁵⁻⁸ The purpose of this study was to identify the impact of pharmaceuticals commonly found in surface waters on immune cells of harbor seals (*Phoca vitulina*) *in vitro*. Peripheral blood mononuclear cells (PBMCs) from captive seals and a seal B lymphoma cell line (11B7501)⁹ were exposed to selected single substances. Compounds of interest were analgesics (ibuprofen, naproxen), psychoactive substances (carbamazepine, paroxetine), antibiotics (erythromycin, sulfamethoxazole, trimethoprim), cholesterol lowering compounds (gemfibrozil), steroidal hormones (ethynyl estradiol) and caffeine.

Materials and Methods

Sampling and isolation of peripheral blood mononuclear cells and cell culture

Whole blood samples (7-15 mL) were collect-

ed from the extradural intervertebral vein of four female harbor seal adults in captivity (Aquarium du Québec, Quebec, Canada). Blood was kept in heparin tubes at RT for 6 h after sampling until separation with Lympholyte-Mammal (Cedarlane, Burlington, Canada). The PBMCs were resuspended in completed RPMI-1640 and kept at 4°C overnight until the start of the incubation with xenobiotics.

The 11B7501 cell line⁹ was maintained at concentrations between 2.0×10^5 - 2.0×10^7 cells/mL in completed RPMI-1640 in a humidified 5% CO₂ atmosphere.

Preparation of xenobiotics

Chemicals (all Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO), ethanol or water. The final concentration of DMSO or ethanol in the samples never exceeded 0.1%.

In vitro exposures

For the *in vitro* exposures, cells were adjusted to the test specific concentration. In case of the lymphoblastic proliferation 200 µL of cells (1.25×10^6 cells/mL) were incubated with 2 µL of pharmaceutical product. In case of phagocytosis and cell cycle and apoptosis, 500 µL of cells (1.0×10^6 cells/mL and 0.5×10^6 cells/mL, respectively) were incubated with 5 µL of pharmaceutical product. Viability assays were set up parallel to each of the three assays as an additional control.

For the experiments with PBMCs, concentrations of pharmaceuticals were based on environmentally relevant values found in municipal effluents. The concentration 1x refers to the maximum concentration detected in the environment (Table 1), as cited.^{10,11} Further concentrations tested were: 0x, 0.01x, 0.1x, 10x, 100x (Table 1).

In experiments with the 11B7501 cell line, concentrations of pharmaceuticals were increased to be able to observe a possible negative impact of the single substance on the harbor seal immune cells (Table 1).

Viability assay

After the respective exposure time, viability of cells was evaluated using 4 µL of a 100 µg/mL of propidium iodide (PI) solution (Sigma-Aldrich) to 500 µL of cell suspension. A FACSCalibur (Becton Dickinson, San Jose, CA, USA) with an air-cooled argon laser providing an excitation at 488 nm was used. For each sample 5 000 events were acquired at a fluorescence emission of 620 nm (FL3). The cell population was electronically gated in a FSC/SSC dot plot and the fluorescence frequency distribution histogram was obtained using FL3. The percentage of dead cells was determined using a marker. Data collection and analysis were performed with the

Correspondence: Christine Kleinert, Institut National de la Recherche Scientifique (INRS)-Institut Armand-Frappier, Laval, H7V 1B7, Canada. E-mail: Christine.Kleinert@iaf.inrs.ca

Key words: immune system, *Phoca vitulina*, pharmaceuticals.

Conference presentation: part of this paper was presented at the *ECOBIM meeting*, 2013 May, Montréal, Quebec, Canada.

Acknowledgements: the study was supported by the Canada Research Chair in Immunotoxicology (MF) and the NSERC Strategic Grant. CK was supported by a scholarship of the German Academic Exchange Service (DAAD). Furthermore, we would like to thank Stéphane Masson and his team at the Aquarium du Québec for providing us with whole blood samples.

This work is licensed under a Creative Commons Attribution NonCommercial 3.0 License (CC BY-NC 3.0).

©Copyright C. Kleinert et al., 2013
Licensee PAGEPress, Italy
Journal of Xenobiotics 2013; 3(s1):e5
doi:10.4081/xeno.2013.s1.e5

CellQuest Pro software (Version 4.0.1). The results were expressed in percentage of viable cells.

Phagocytosis assay

Phagocytosis was measured using carboxylate coated fluorescent latex beads (Polysciences Inc.; 100 beads:1 cell). After 24 h exposure to single substances of pharmaceuticals, cells and beads were incubated for 1.5 h at 37°C to allow attachment and phagocytosis. To remove most free beads after incubation, the suspension was centrifuged on a gradient of 3% BSA (MP Biomedicals) prepared in completed medium. The fluorescence emission was collected at 520 nm (FL1). For each sample 10,000 events were acquired. Using a FSC/SSC dot plot, the adequate population was electronically gated and the fluorescence frequency distribution histogram was obtained using FL1. Data was expressed as percentage of cells with phagocytic activity (cells that engulfed >one bead) and phagocytotic efficiency (cells that engulfed >three beads). The lymphocyte population was gated as a negative control.

Lymphocyte transformation assay

Change in DNA synthesis after stimulation with a mitogen [Con A for PBMCs, lipopolysaccharide (LPS) for 11B7501 cell line] was measured as the incorporation of methyl-³H-thymidine. After 48 h incubation, 1 µCi of methyl-³H-thymidine (PerkinElmer, Shelton, USA) was

added and cells were incubated for further 18 h. The cells were harvested onto a glass fiber filter (Tomtec Mach III Cell Harvester) and the amount of radioactivity incorporated was measured with a TriLux counter (Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer) and analyzed with the program MicroBeta Windows Workstation (Version 4.50.09, PerkinElmer, Shelton, USA). The raw data was expressed as counts per minute and was then converted in percent proliferation of control.

Cell cycle and apoptosis assay

The DNA content of each cell was measured using PI. After fixation of the cells with 70% EtOH following the 24 h exposure and 72 h in RPMI, cells were washed and then resuspended in a PBS solution containing PI (50 µg/mL) and RNase (100 µg/mL) (all Sigma-Aldrich). PI also binds to double stranded regions of RNA, necessitating treatment with nucleases.¹² Using the FSC/SSC dot plot, the cell population was electronically gated and the fluorescence frequency distribution histogram using FL2 (585 nm) was obtained. In a second dot plot, the gated lymphocyte population is expressed in FL2-A/FL2-W. Doublets (two G₀/G₁ cells attached to each other, which seem to have the same DNA content as one cell in the G₂/M phase) are discriminated using a second gate. The population *lymphocytes minus doublets* was analyzed in FL3-A, and apoptosis as well as the phases of the cell cycle were gated independently. For each sample 5000 events were acquired. The results were expressed in percentage of cells in different stages of the cell cycle plus apoptotic events.

Statistical analyses

Differences between controls and treated groups were evaluated by one-way ANOVA followed by Tukey's Multiple Comparison post test. The calculations were performed using GraphPad Prism 5 for Windows (GraphPad Software). The level of significance was set at $P \leq 0.05$.

Results and Discussion

The experiments conducted with PBMCs exposed to concentrations of pharmaceuticals similar to those found in surface showed no significant impairment of cellular function in the phagocytosis or lymphoblastic proliferation assay (*data not shown*).

In the experiments with the 11B7501 cell line, three compounds influenced the B lymphocytes at high concentrations. While the phagocytosis experiment showed no significant effect with any compound (*data not*

shown), the lymphoblastic proliferation with LPS was modulated in the case of naproxen (>25 µg/mL**) and carbamazepine (>50 µg/mL*) (Figure 1A). The cell cycle and apoptosis assay revealed a significant increase in apoptotic events at 50 µg/mL for ethynylestradiol, while the percentage of cells in the G₂/M phase

decreased (Figure 1B). For carbamazepine, the percentage of apoptotic events slightly decreased at 50 µg/mL, while the percentage of cells in the G₀/G₁ phase increased (Figure 1B).

The absence of an effect in the lower doses of pharmaceutical compounds does not neces-

Table 1. Concentration range of pharmaceutical compounds tested with PBMCs and the 11B7501 cell line. For the experiments with PBMCs, test concentrations of single substances were chosen according to values found in municipal effluents. The maximum value found in literature was set to be the 1x concentration. Further concentrations tested were 0x, 0.01x, 0.1x, 10x and 100x of the 1x value. Concentration ranges of the single substances for the experiments with the 11B7501 cell line were then increased.

Compound	PBMCs		11B7501
	1x (µg/L)	Source	
Caffeine	800	Verlicchi <i>et al.</i> , 2010 ¹¹	0.125-25
Ibuprofen	300	Verlicchi <i>et al.</i> , 2010 ¹¹	0.125-25
Gemfibrozil	30	Verlicchi <i>et al.</i> , 2010 ¹¹	0.25-50
Naproxen	20	Verlicchi <i>et al.</i> , 2010 ¹¹	0.25-50
Sulfamethoxazole	12	Terzic <i>et al.</i> , 2008 ¹⁰	0.25-50
Trimethoprim	6	Verlicchi <i>et al.</i> , 2010 ¹¹	0.25-50
Carbamazepine	2	Verlicchi <i>et al.</i> , 2010 ¹¹	0.25-50
Erythromycin	0.4	Terzic <i>et al.</i> , 2008 ¹⁰	0.25-50
Ethynylestradiol	-	-	0.25-50
Paroxetine	-	-	0.25-50

PBMCs, peripheral blood mononuclear cells.

A) Lymphoblastic proliferation with lipopolysaccharide

B) Cell cycle and apoptosis

Figure 1. The lymphoblastic proliferation assay revealed significant effects for naproxen and carbamazepine (A). The cell cycle and apoptosis assay showed significant effects for ethynyl estradiol and carbamazepine (B). Significant differences are expressed by an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

sarily conclude to the harmlessness of current environmentally relevant levels of pharmaceuticals towards the immune system of marine vertebrates. In contrast, many studies on fish already showed bioaccumulation in various tissues and a significant change in immune parameters, behavior and distribution that were caused by environmentally relevant levels of pharmaceuticals.¹³⁻¹⁶ With fish accumulating pharmaceutical products and seals being piscivorous, they will likely be exposed to higher concentrations through nutrition, opposed to those concentrations found in the water column. Therefore, the true environmentally relevant concentrations for the exposures of marine mammals towards pharmaceuticals still have to be assessed.

Our study shows that more sensitive methods are needed to evaluate the impact of pharmaceuticals on the immune system of marine mammals. Risk assessment in this area is necessary, because not only seals but also humans consume fish. If pharmaceuticals in the environment cause a possible reduction in certain functional activities of the immune system of large vertebrates that may alter the host's resistance to pathogens, the knowledge of these effects would therefore most certainly have implications on the treatment of municipal wastewaters in the future.

References

1. Togola A, Budzinski H. Analytical development for analysis of pharmaceuticals in water samples by SPE and GC-MS. *Anal Bioanal Chem* 2007;388:627-35.
2. Gagné F, Blaise C, Fournier M, Hansen PD. Effects of selected pharmaceutical products on phagocytic activity in *Elliptio complanata* mussels. *Comp Biochem Phys C* 2006;143:179-86.
3. Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE. Occurrence, fate and effects of pharmaceutical substances in the environment - A review. *Chemosphere* 1998; 36:357-94.
4. Sanderson H, Johnson DJ, Reitsma T, Brain RA, Wilson CJ, Solomon KR. Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. *Regul Toxicol Pharm* 2004;39:158-83.
5. Jones OAH, Voulvoulis N, Lester JN. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. *Water Res* 2002;36:5013-22.
6. Sanderson H, Johnson DJ, Wilson CJ, Brain RA, Solomon KR. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. *Toxicol Lett* 2003;144:383-95.
7. Stuer-Lauridsen F, Birkved M, Hansen LP, Lützhøft HCH, Halling-Sørensen B. Erratum to "Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use" [*Chemosphere* 40 (2000) 783-793]. *Chemosphere* 2000;41:1509.
8. European Commission. White paper. Strategy for a future chemicals policy, COM (2001) 88 Final. Available from: http://ec.europa.eu/enterprise/sectors/chemicals/documents/reach/archives/white-paper/index_en.htm Accessed: 12 Nov 2012.
9. Frouin H, Fortier M, Fournier M. Toxic effects of various pollutants in 11B7501 lymphoma B cell line from harbour seal (*Phoca vitulina*). *Toxicology* 2010;270:66-76.
10. Terzić S, Senta I, Ahel M, Gros M, Petrović M, Barcelo D, et al. Occurrence and fate of emerging wastewater contaminants in Western Balkan Region. *Sci Total Environ* 2008;399:66-77.
11. Verlicchi P, Galletti A, Petrović M, Barcelo D. Hospital effluents as a source of emerging pollutants: An overview of micropollutants and sustainable treatment options. *J Hydrol* 2010;389:416-28.
12. Suzuki T, Fujikura K, Higashiyama T, Takata K. DNA staining for fluorescence and laser confocal microscopy. *J Histochem Cytochem* 1997;45:49-53.
13. Hoeger B, Hitzfeld B, Kollner B, Dietrich DR, van den Heuvel MR. Sex and low-level sampling stress modify the impacts of sewage effluent on the rainbow trout (*Oncorhynchus mykiss*) immune system. *Aquat Toxicol* 2005;73:79-90.
14. Salo HM, Hébert N, Dautremepuits C, Cejka P, Cyr DG, Fournier M. Effects of Montreal municipal sewage effluents on immune responses of juvenile female rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* 2007;84:406-14.
15. Azzurro E, Matiddi M, Fanelli E, Guidetti P, La Mesa G, Scarpato A, et al. Sewage pollution impact on Mediterranean rocky-reef fish assemblages. *Mar Environ Res* 2010; 69:390-7.
16. Daughton CG, Brooks BW. Chapter 8: Active pharmaceutical ingredients and aquatic organisms. In: Beyer WN, Meador JP, eds. *Environmental contaminants in biota: interpreting tissue concentrations*. Boca Raton, FL: CRC Press, Taylor and Francis; 2011. pp 286-347.