Cell-based assay detects residual nonsteroidal anti-inflammatory drugs (NSAIDs) in effluent of municipal wastewater treatment

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- *In vitro* mode-of-action-based assay detects NSAIDs by their COX-inhibiting function
- Detection of residual pharmaceuticals in wastewater treatment plant effluents
- CLARIOstar® microplate reader measures ratiometric probe using the LVF Monochromator™

Introduction

Residues of human pharmaceuticals, such as NSAIDs and their metabolites, are increasingly found in effluents of municipal wastewater treatment plants (WWTP) all over Europe. NSAIDs reduce pain and inflammation by inhibiting cyclooxygenases (COX) and thereby the production of prostanoids such as prostaglandins. The amino acid sequence of COX targeted by NSAIDs is evolutionarily highly conserved among vertebrates. Thus, it appears likely that organisms expressing COX, also physiologically respond to NSAIDs when exposed to them. For risk assessment one has to know the extent to which aquatic organisms are exposed to NSAIDs as well as metabolites with the same mode of action. Therefore, we developed and validated a cell-based mode-of-action (MOA) assay, by which the total NSAID activity in a wastewater sample enriched by solid phase extraction (SPE) can be measured as equivalents of the lead substance diclofenac (DicEQ).

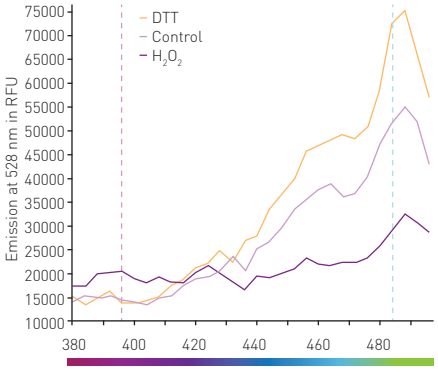

Fig. 2: The redox sensor Grx-roGFP3: When reduced by DTT (organe), fluorescence of Grx-roGFP3 increases when excited at 485nm and decreases when excited at 395nm. Oxidation by H_2O_2 (dark purple) reverses the situation. Light purple line shows unperturbed cells.

Assay Principle

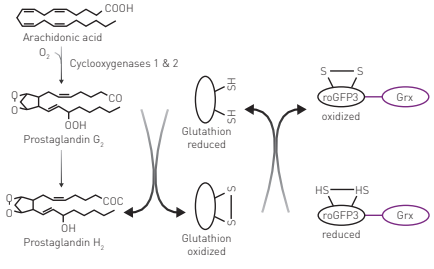

Fig. 1: NSAID biosensor cell line: In absence of NSAID, COX catalyzes the reaction of the substrate arachidonic acid to reactive intermediate lipid peroxides. These can be detected via the Grx-roGFP3 redox sensor.

Materials & Methods

- CHO cells (DSMZ-No. ACC-110)
- Microplate (96-well, black bottom, GREINER)
- Diclofenac (Cayman Chemical Company)
- Arachidonic acid (Sigma)
- Plasmids (SIZ Zellkulturtechnik Mannheim)
- CLARIOstar microplate reader (BMG LABTECH)

Experimental procedure

CHO cells were cotransfected with the genetically encoded fluorescent redox sensor Glutaredoxin-(Grx-) roGFP3 and COX1 using FUGENE-HD (Promega). Upon selection with G418 and hygromycin, cells were expanded, subcloned and screened for COX1 activity [1]. The COX1-dependent oxidation of the redox sensor Grx-roGFP3 increases the excitation fluorescence ratio 395nm/485nm, while NSAIDs concentration-dependently limit the increase. The Grx-roGFP3-COX1 sensor cell line was used to assay for NSAID activities in wastewater samples. Measurements were done with the CLARIOstar plate reader using the settings indicated below. Activities were measured in diclofenac equivalents (DicEQ).

Instrument settings

Optic settings	Fluorescence intensity, well mode kinetic	
	Top optic	
	No. of multichromatic	2
	Monochromator settings	Oxidized roGFP Excitation: 395-15 Dichroic: 460.2 Emission: 528-20 Gain: 2500
		Recuced roGFP Excitation: 485-15 Dichroic: 505.2 Emission: 528-20 Gain: 1800
	Focal height	4.7 mm
Well scan	Orbital averaging	Diameter: 4 mm

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Kinetic settings	No. of flashes	20
	Number of intervals	3 (kinetic window 1) 25 (kinetic window 2)
	Interval time	5 s
Injection	Injection time	After 4th cycle
	Injected volume	40 µL
	Pump speed	430 µl/s
	Shake after injection	1 s at 300 rpm, double orbital

Results & Discussion

Exposing roGFP3- and COX1- expressing cells to WWTP effluents decreases the roGFP ratio as a result of COX1 inhibition (Fig. 3).

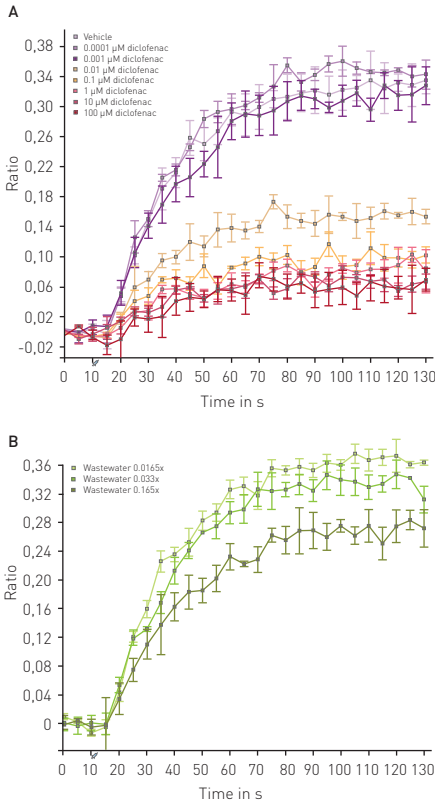

Fig. 3: NSAID assay in SPE-enriched wastewater sample: Response curves of lead substance diclofenac **(A)** and SPE-enriched wastewater sample **(B)**, each at different concentrations, are shown. Wastewater samples were measured in different concentrations. Substrate arachidonic acid was added after the third measurement.

The assay was validated by the lead substance standard diclofenac of known quantity. The NSAID activity of the assayed wastewater sample was found to be 3.5 µg/l DicEQ (Fig. 4). Corresponding diclofenac concentration determined by chemical analysis (LC-MS/MS) was 2.2 µg/l (TZW Karlsruhe, data not shown). The higher NSAID activity measured by the *in vitro* assay of SPE-enriched wastewater compared to the diclofenac concentration determined by chemical analysis is explained by the presence of further NSAIDs. A chemical analysis of selected pharmaceutical compounds determined additional NSAIDs like ibuprofen and naproxen in the wastewater sample (TZW Karlsruhe, data not shown).

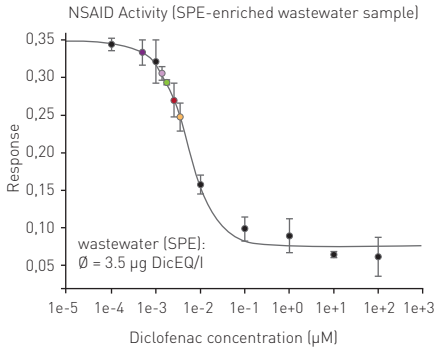

Fig. 4: NSAID activity in SPE-enriched wastewater sample: Concentration-response curve was generated from lead substance diclofenac at different concentrations (black dots). From this grading curve NSAID activity in SPE-enriched wastewater sample was determined at different concentrations (coloured dots) and calculated as µg diclofenac equivalent (DicloEQ) per l.

Conclusion

The MOA-based NSAID assay in living cells allows for measuring total NSAID activities in complex mixtures such as WWTP effluents as equivalents of the lead substance diclofenac (DicEQ). Further NSAIDs present in the WWTP effluent inhibited COX1 as confirmed by chemical analysis. The assay can be devised as a standard method with the potential for routine employment in large-scale monitoring of WWTP effluents and the aquatic environment they are discharged into. The CLARIOstar multi-mode microplate reader reliably detected the ratiometric roGFP probe using its unique LVF monochromator™. Its flexibility to record fluorescence spectra supported development of the NSAID assay.

References

1. Bernhard et al. (2017) Water Research **Vol. 115**, pp. 74-83, May 15, 2017. doi: 10.1016/j.watres.2017.02.036