INHIBITION OF P-GP EFFLUX PUMPS AND SUPRAMAXIMAL EFFECT (SPMX) ON *IN VITRO* DIOXIN BIOASSAY

Montaño M[1]*, Hoffmann L[1], Gutleb AC[1], Murk AJ[2]

[1]Centre de Recherche Public – Gabriel Lippmann, Department Environment and Agro-biotechnologies, 41, rue du Brill, L-4422 Belvaux, Grand Duchy of Luxembourg; [2]Section of Toxicology, Wageningen University, NL-6700 EA, P.O. Box 8000, Wageningen, The Netherlands.

Introduction

In vitro bioassays are increasingly applied to complex extracts to relatively quantify estrogenic, androgenic, progestagenic and dioxin-like activity. A casual feature of these bioassays is the "supramaximal effect" (SPMX), occurring when the response induced by the tested compound is significantly higher compared to the natural ligand [1]. This effect has been observed after incubation with some pure substances on estrogenic receptor (ER) mediated assays [1], but also after incubation with complex mixtures like oils [2], oil contaminated pore water [3], and sediments [4, 5] on dioxin receptor (DR) mediated luciferase reporter gene assays. The causes of this effect are still uncertain; a recent literature study of estrogenic SPMX effect inducers pointed towards the characteristics of the transfected cell lines [1], while a mechanistic study with genistein on an ER-mediated luciferase assay suggested a post-transcriptional stabilization of the firefly luciferase reporter enzyme [6].

The multi-xenobiotic resistance (MXR) is a phenomenon in which over-expression of cellular efflux proteins results in enhancement of protection of organism against environmental contaminants [7]. Cellular efflux pumps like P-glycoprotein (P-gp also known as MDR1) are expressed in peripheral and barrier tissues of several marine organisms [8]. Diverse environmental contaminants inhibit the MXR mechanism *in vitro* [9], and *in vivo* [10], and some of these contaminants potentiate the toxic effect of an efflux pumps substrate [10].

P-gp is present in significant amounts in the rat liver hepatoma cell line H4IIE [11], which is a common cell line used in the DR-mediated luciferase reporter gene assay (DR.Luc) [5]. We therefore hypothesized that P-gp efflux pump blockers will potentiate the effect of a dioxin-like agonist and perhaps induce a SPMX effect on the H4IIE based DR.Luc assay. To test this hypothesis, we adapted the 96-well plate cellular efflux pump inhibition assay (CEPIA) [9] to the H4IIE.Luc cell line, and measured the inhibition potency of verapamil as model P-gp inhibitor, pentachlorophenol (PCP) and 2,3,7,8-tetrachlorodibenzo-p-dioxine (TCDD). Then we tested verapamil and various environmentally relevant efflux pump inhibitors, alone and in co-exposure with TCDD on the DR.Luc assay.

Materials and methods

Compounds were >98% pure and purchased from Sigma-Aldrich, except for PCP and 4'-OH-3,3',4,5'-CB 79 (4'-OH CB 79) which were purchased from Accustandard and LGC Promochem, respectively. All compounds were dissolved in DMSO and then diluted in phosphate buffer saline (PBS) for the CEPIA assay or cellular media for the DR.Luc assay, with a final DMSO concentration of 0.2% (v/v) for single exposures and 0.4% (v/v) for co-exposures. H4IIE.Luc cells were a kind gift from Prof. Michael Denison and were cultured and plated for assays as previously published [2].

Adaptations of the CEPIA assay on H4IIE.Luc cells were based on the previously standardized method by Georgantzopoulou and co-workers [9]. Calcein-AM is a non fluorescent P-gp substrate which can be metabolized by cytosolic esterases into the fluorophore calcein. Calcein is not a P-gp substrate, thus upon P-gp inhibition calcein will accumulate inside the cell and increase the fluorescence response [9]. Cells were incubated for 30 min with 100 µL of PBS alone or with test compounds. Then they were supplemented with calcein-AM as a model substrate at final concentrations between 0.05 and 5 µM in 100 µL of PBS, and incubated for 15 to 180 min. Fluorescence was measured in a Biotech Synergy 2 plate reader at 485 nm excitation and 530 nm emission wavelengths. Standard curves were finally obtained with 3 µM calcein-AM exposure for 45 min.

For the DR.Luc assay, H4IIE.Luc cells were exposed to TCDD standards (0.2% DMSO v/v), P-gp pump inhibitors alone and in combination with TCDD at the EC_{50} and maximum levels (final conc. 10 and 100 pM respectively, 0.4% DMSO v/v) in 100 µL of fresh media for 24 hours. Inhibitor concentrations were chosen according to their *in vitro* CEPIA inhibitory concentration [9]. Viability was determined by the measurement of the cells' metabolic activity with 4 µM resazurin in 200 µL cell media for 3 hours [12], immediately followed by the luciferase analysis on the same plate as previously published [2]. Both measurements were performed in a Biotech Synergy 2 plate reader.

All experiments were performed in triplicate and repeated twice. Data points represent mean ± SD of relative fluorescence units (RFU) or luminescence units (RLU), relative to TCDD maximum response at 100 nM minus DMSO control. The dose response data were fitted to a Hill equation with SigmaPlot 2001 (SPSS Inc.), and 50% inhibitory concentrations (EC_{50}) were calculated from the modeled data. Statistical analysis was performed with ANOVA followed by Duncan (Figures 1A and 1B) or Tukey post-hoc comparison ($P<0.01$) (SPSS, Inc).

Results and Discussion

H4IIE.Luc CEPIA assay. First, the optimal conditions for the CEPIA assay with H4IIE.Luc cells were determined. After incubation of H4IIE.Luc cells with calcein-AM, a significant increase in fluorescence was observed after 180 min with 0.5-1 µM, after 60 min with 2 µM or after 15 min above 3 µM (Figure 1A). The background fluorescence observed without inhibitor is likely due to calcein-AM hydrolysis before it is withdrawn from the cell. Incubation with the P-gp inhibitor verapamil (100 µM) induced a significant increase in fluorescence after 180 min with 0.5 µM calcein-AM, and after 15 min above 1 µM (Figure 1B). A concentration of 3 µM calcein-AM for 45 min was chosen to obtain a signal sufficiently high to properly observe inhibition.

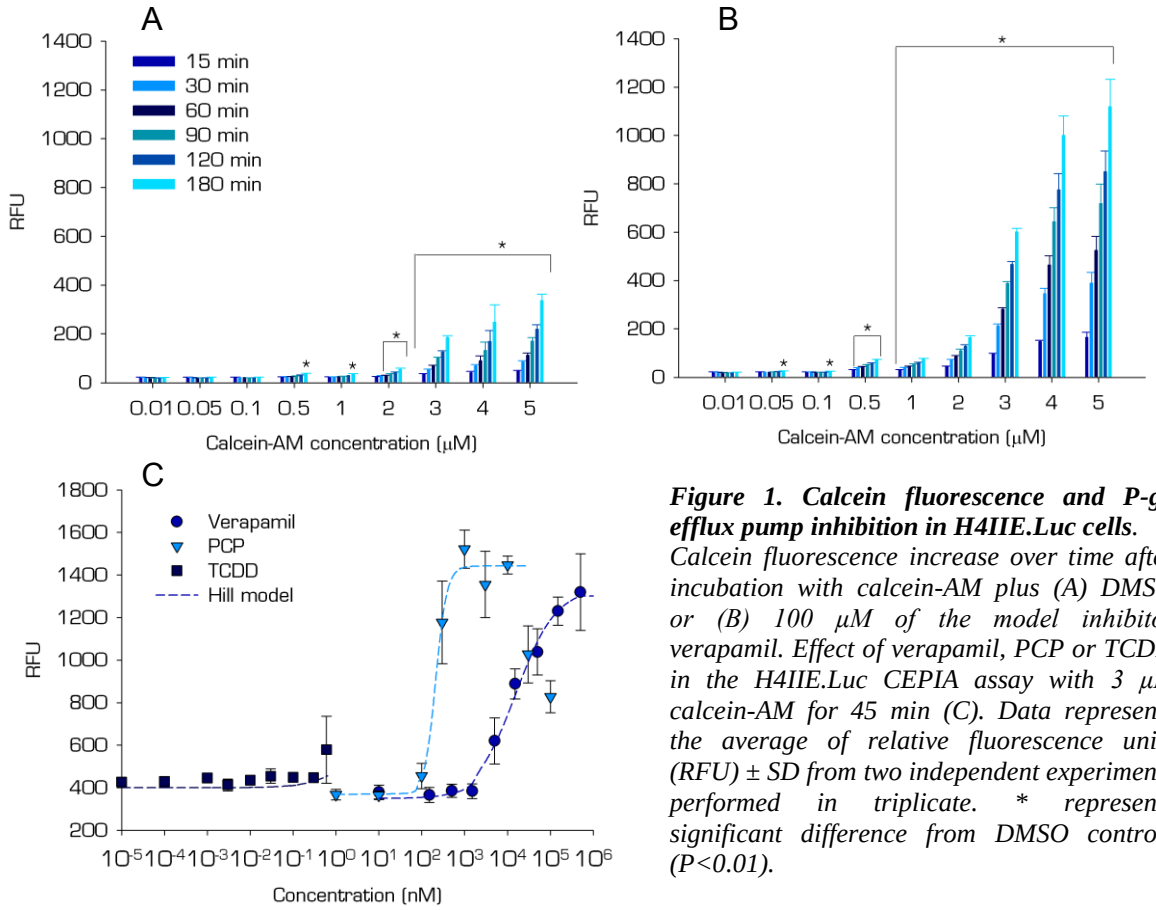

Figure 1. Calcein fluorescence and P-gp efflux pump inhibition in H4IIE.Luc cells. *Calcein fluorescence increase over time after incubation with calcein-AM plus (A) DMSO or (B) 100 µM of the model inhibitor verapamil. Effect of verapamil, PCP or TCDD in the H4IIE.Luc CEPIA assay with 3 µM calcein-AM for 45 min (C). Data represents the average of relative fluorescence units (RFU) ± SD from two independent experiments performed in triplicate. * represents significant difference from DMSO controls (P<0.01).*

The P-gp inhibition of verapamil, PCP and TCDD was then evaluated with the CEPIA assay optimized for H4IIE.Luc cells. Verapamil and PCP produced a dose-related increase in calcein fluorescence, while TCDD did not significantly increase the fluorescence compared to the DMSO controls (Figure 1C). The significant reduction in fluorescence observed above 30 µM PCP was due to cytotoxicity which was visually confirmed under the light microscope and by the cell viability assay (data not shown). The EC_{50} of verapamil and PCP were 16±2.5 µM and 0.21±0.02 µM, respectively, which was 3-6 times lower compared to inhibitory concentrations reported for P-gp over-expressing Madin-Darby canine kidney MDCKII-MDR1 cells [9]. TCDD is known to induce P-gp mRNA expression [13] but to the best of our knowledge it is not known whether TCDD is a P-gp substrate. Up to 600 nM TCDD did not inhibit Ca-AM efflux, a concentration which is far above the EC50 used for mixture toxicity studies causing SPMX effects [2].

DR.Luc assay. First, the effect of PCP on the TCDD AhR-mediated response was evaluated in the DR.Luc assay. TCDD produced a dose-related response in the AhR-mediated DR.Luc assay with a limit of detection of 1.3±2.8 pM, an EC_{50} of 14±2.0 pM and an ECmax at 100 nM (Figure 1A). These parameters are comparable to previously reported standardized results [14]. DMSO and the PCP concentration that gave maximal P-gP inhibition (1 µM PCP), were added to a full TCDD dose-response curve (0.4% DMSO) and compared with TCDD alone (0.2% DMSO). If considered isolated, 1 µM PCP induced a significant increase of TCDD luciferase production at concentrations above 100 pM (Figure 1A). However, the overall effect of DMSO or PCP compared to TCDD alone was not significant, as the effect of TCDD plus 1 µM PCP compared to TCDD plus DMSO was also not significantly different (ANOVA, P>0.01).

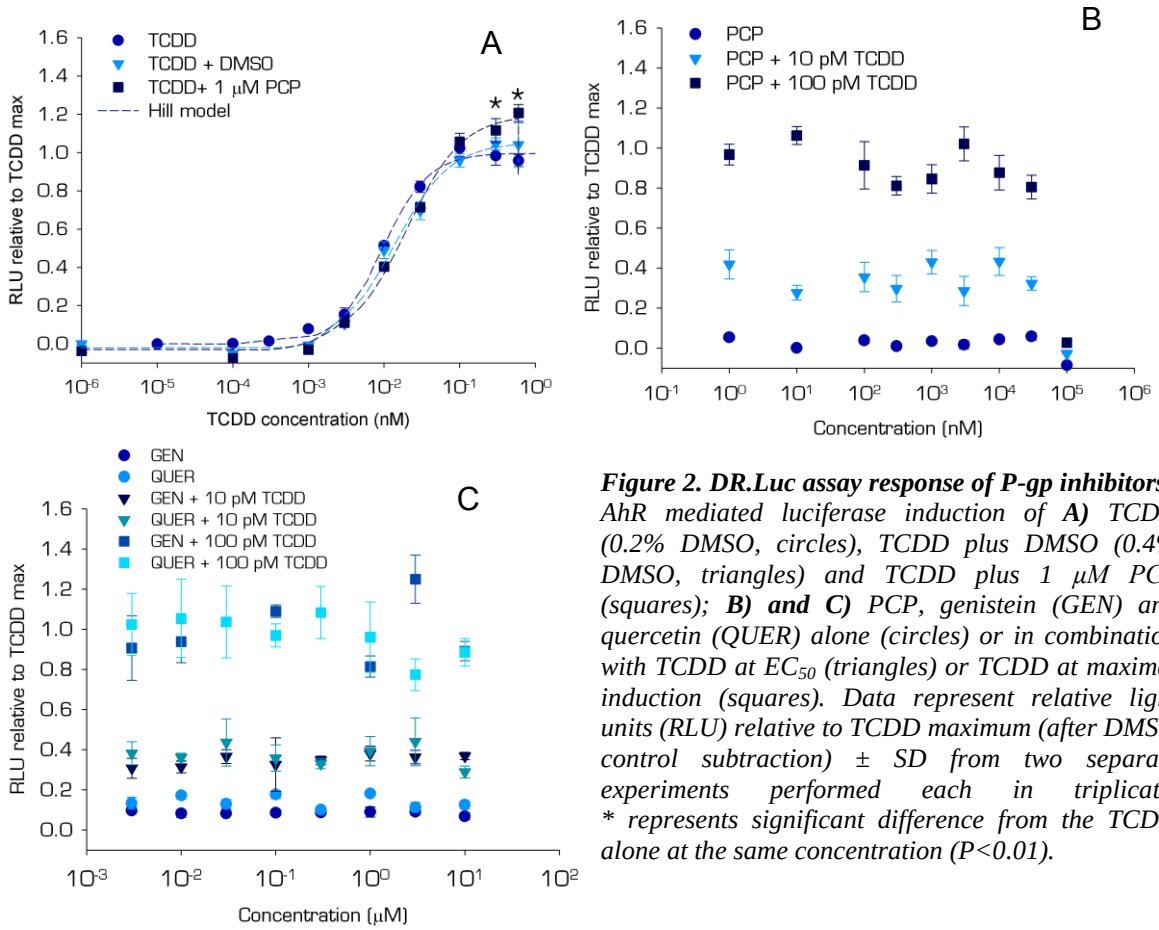

Figure 2. DR.Luc assay response of P-gp inhibitors*. AhR mediated luciferase induction of* ***A)*** *TCDD (0.2% DMSO, circles), TCDD plus DMSO (0.4% DMSO, triangles) and TCDD plus 1 µM PCP (squares);* ***B) and C)*** *PCP, genistein (GEN) and quercetin (QUER) alone (circles) or in combination with TCDD at* EC_{50} *(triangles) or TCDD at maximal induction (squares). Data represent relative light units (RLU) relative to TCDD maximum (after DMSO control subtraction) ± SD from two separate experiments performed each in triplicate. * represents significant difference from the TCDD alone at the same concentration (P<0.01).*

PCP did not induce a dioxin-like response alone, nor increased the response of 10 or 100 pM TCDD (Figure 2B). Verapamil, bisphenol-A, genistein, quercetin, indol-3 butiric acid, indol-3-acetic acid, and 4-OH CB 79 were tested alone and in co-exposure in the DR.Luc assay with similar negative results (data not shown). There was therefore no evidence that inhibitors of P-gp efflux pumps, at the concentrations tested, could induce luciferase production or potentiate that of TCDD on the DR.Luc assay.

Genistein and quercetin are known for inducing the SPMX effect in the ER-mediated reporter gene assays with more than 2 ERE's in the reporter construct [1]. Quercetin has been reported to inhibit P-gp activity [9]. However, both failed to induce AhR-mediated luciferase activity or to increase the response induced by 10 or 100 pM TCDD (Figure 2C). Therefore the proposed mechanism of post-transcriptional luciferase stabilization [6] does not apply to luciferase produced in the H4IIE.Luc cells and might not be the most important mechanism behind the SPMX effect.

Conclusions. The CEPIA assay was successfully adapted to the H4IIE.Luc cell line. The model P-gp inhibitor verapamil and PCP were more potent in this cell line compared to results obtained with the P-gp over-expressing MDCKII-MDR1 cell line. Under the DR.Luc assay conditions there was no evidence that P-gp efflux pump inhibitors modified or potentiated the activity of TCDD. Neither genistein nor quercetin, two potent SPMX inducers on ER-mediated assays, induced any signal on the DR.Luc assay, nor influenced the luciferase induction by TCDD. Future work should be focused on testing the consequences of efflux pump inhibition with an AhR-agonist which is a P-gp substrate, as this could result in intracellular accumulation of this AhR-agonist.

Acknowledgements
The REARISK project (M.M.) is funded by the Fonds National de la Recherche (FNR) Luxembourg (TR-PHD-BFR08 098).

References:

1. M. Montano, E. J. Bakker, A. J. Murk. (2010) *Toxicol. Sci.* 115(2): 462-474.
2. C. M. Vrabie, M. T. O. Jonker, A. J. Murk. (2009) *Environ. Toxicol. Chem.* 28(9): 1995-2003.
3. M. T. O. Jonker *et al.* (2006) *Environ. Toxicol. Chem.* 25(5): 1345-1353.
4. D. S. Baston, M. S. Denison. (2011) *Talanta* 83(5): 1415-1421.
5. A. J. Murk *et al.* (1996) *Fundam. Appl. Toxicol.* 33(1): 149-160.
6. A. M. Sotoca *et al.* (2010) *J. Steroid Biochem. Mol. Biol.* 122(4): 204-211.
7. S. M. Bard. (2000) *Aquatic Toxicol.* 48(4): 357-389.
8. B. Kurelec *et al.* (2000) *Ecotoxicol.* 9(5): 307-327.
9. A. Georgantzopoulou *et al.*, Submitted.
10. H. Anselmo *et al.*, Submitted.
11. R. Mehta *et al.* (1994) *Cancer Letters* 84(2): 163-172.
12. M. Montaño *et al.* (2011) *Food Chem. Toxicol.* 49(9): 2328-2335.
13. J. M. Maher *et al.* (2005) *Drug Metab. Dispos.* 33(7): 956-962.
14. H. T. Besselink *et al.* (2004) *Environ. Toxicol. Chem.* 23(12): 2781-2789.