Application of the PPARγ$_2$-CALUX® Assay to the Biological Detection of Endocrine Active Chemicals

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SUMMARY

Obesity and the related metabolic diseases have increased drastically over the past decades, becoming a global health crisis. Recently, scientists have begun to suspect the role of environmental chemicals in the obesity epidemic, forming the hypothesis of 'obesogens', endocrine active compounds which may promote obesity by inappropriately regulating lipid metabolism and adiopgenesis.

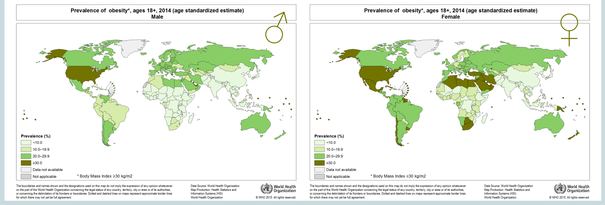


Figure 1: WHO reported prevalence of adult obesity worldwide in 2014

A key target of obesogens could be the peroxisome proliferator-activated receptor γ (PPARγ) because of its role in the induction of adipogenesis. PPARγ can be perturbed by a wide spectrum of environmental chemicals due to its large ligand-binding pocket; this makes PPARγ a vulnerable target for endocrine active substances (EASs). Screening for PPARγ-active compounds has become a priority; *in vitro* screening tools have several advantages among the assays that can be used to detect a disruption in PPARγ activity. The PPARγ$_2$-CALUX® assay is one method of choice and was implemented in-house at Nestlé and tested for suitability for inclusion in a battery of tests used for the *in vitro* screening of food-related materials including packaging.

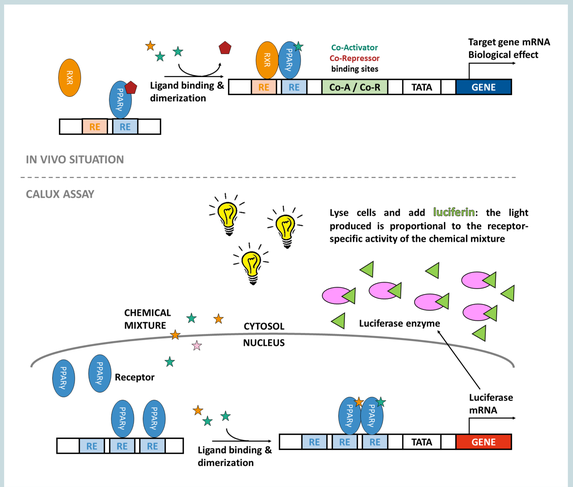


Figure 2: Overview of the PPARγ$_2$-CALUX® reporter gene assay

While *in vitro* models can hardly represent the full levels of toxicokinetic complexity that occur within an organism, for the purposes of biodetection, it is possible to partially overcome this lack of complexity in the PPARγ$_2$-CALUX® assay integrating a liver S9 metabolism protocol into the assay. The PPARγ$_2$-CALUX® assay was successfully implemented in house; results obtained on a number of compounds demonstrated that it performed in agreement with the literature. However, application of the S9 metabolizing protocol on the PPARγ$_2$-CALUX® assay failed to induce changes in the response of tested compounds; in silico data suggested that phase I metabolism of a ligand is not expected to significantly influence its binding to the PPARγ active site.

The CALUX assay was later applied to a set of compounds from the BPx family because of their suspected obesogen effects. Our results revealed antagonistic potential for all compounds (BPA, BPF, BPS, TCBPA, TBBPA, BADGE and BFDGE), but surprisingly only TCBPA and TBBPA were able to activate PPARγ-dependent transcription. Other modes of obesogenic action of chemicals, which are not detected by this assay, may be more important than previously suspected. Therefore, more understanding of the field is necessary before considering this assay as a first priority in routine screening *in vitro* test battery.

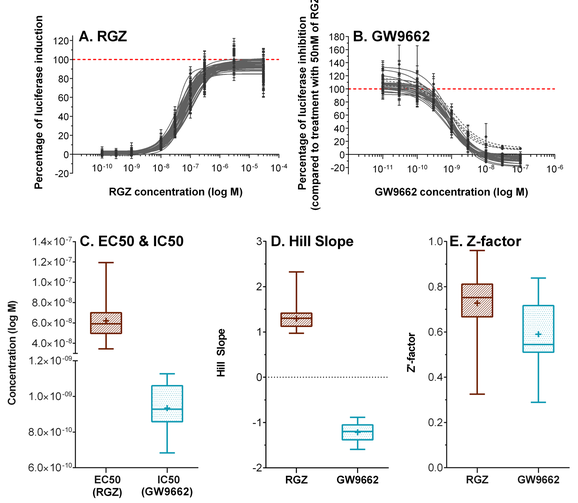
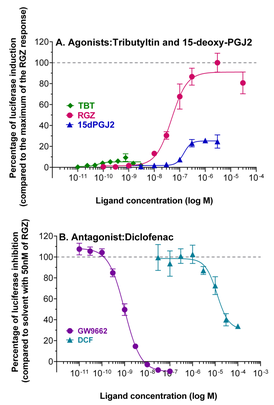


Figure 3: Dose-response curves for the two reference compounds indicate a high-degree of PPARγ$_2$-CALUX® assay reproducibility. All the dose-response curves for RGZ (49 curves; panel A) and GW9662 (27 curves; panel B) obtained during this investigation were combined to verify the reproducibility of the PPARγ$_2$-CALUX® assay. For each curve, the RLU are corrected for solvent control (except where indicated for GW9662 by dotted lines) and normalized such that the mean maximal RLU measured (RGZ) or the average of the 50 nM RGZ-only control wells (GW9662) was set to 100%. All data are expressed as means ± SD (n=3). From these data, values for EC_{50}, IC_{50}, Hill slopes and Z-factors were calculated and plotted as box plots representing 25th and 75th percentiles (box), min and max (end of whiskers), median (—) and mean (+) of 49 RGZ and 27 GW9662 experiments.

Figure 4: Known agonists of PPARγ$_2$, tributyltin (TBT) and 15-deoxy-prostaglandin J2 (15dPGJ2), and the known antagonist diclofenac (DCF) exhibited similar results in the PPARγ$_2$-CALUX® assay to those reported in the literature. Increasing concentrations of TBT and 15PGJ2 were tested in agonist mode (Panel A) in comparison to rosiglitazone (RGZ), the reference agonist. Similarly, increasing concentrations of DCF were tested for their ability to antagonize the effects of 50 nM of RGZ in the antagonist mode of the PPARγ$_2$-CALUX® assay (Panel B) in comparison to GW9662, the reference antagonist. All agonistic or antagonistic activities measured were similar to those obtained in various PPARγ models, confirming the accuracy of the assay.



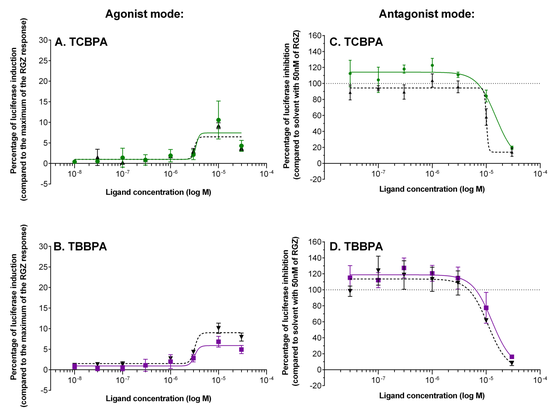


Figure 5: Halogenated bisphenol A derivatives have PPARγ$_2$ agonist and antagonist activities in the PPARγ$_2$-CALUX® assay, indicating probable partial agonism. Increasing concentrations of TCBPA and TBBPA were tested in both agonist (Panels A and B) and antagonist modes in the presence of 50 nM of RGZ (Panels C and D). Each test was performed both in the presence (dotted lines) or absence (plain lines) of S9 metabolizing system. The agonistic activities measured above the LOQ of the test system and all the antagonistic activities observed occurred at concentrations above 10 µM, probably indicating weak partial agonist activity.

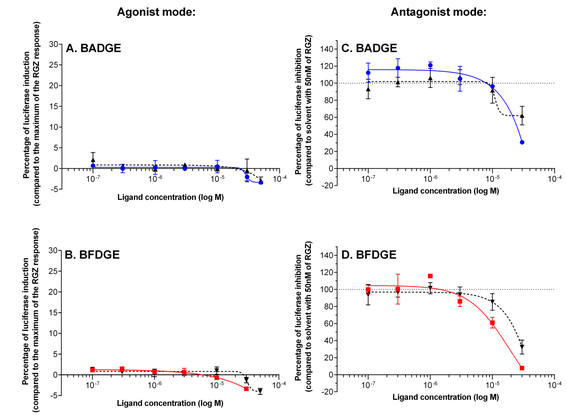


Figure 7: Diglycidyl ether derivatives of bisphenols have no PPARγ$_2$ agonist activity in the PPARγ$_2$-CALUX® assay, but they are PPARγ$_2$ antagonists. Increasing concentrations of BADGE and BFDGE were tested in both agonist mode (Panels A and B) and antagonist mode in the presence of 50 nM of RGZ (Panels C and D). Each test was performed in both the presence (dotted lines) or absence (plain lines) of S9 metabolizing system. All agonistic activities measured were below the LOQ of the test system, indicating no effect. All antagonist activities measured occurred above 10 µM, indicating weak antagonism.

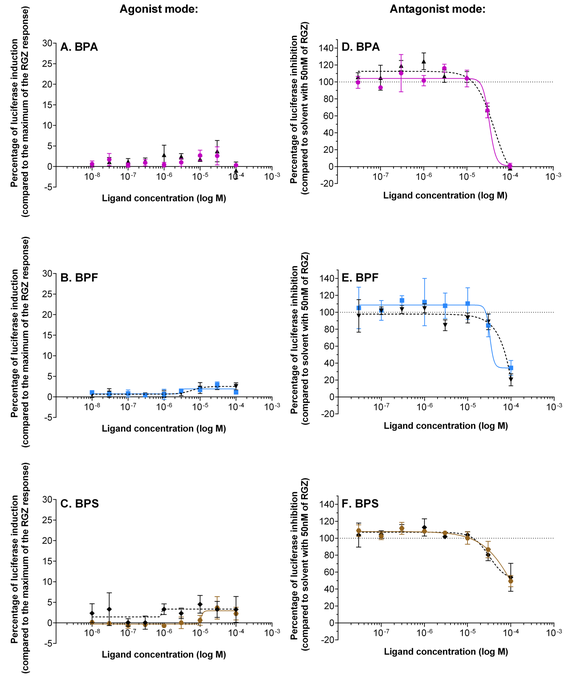


Figure 6: The bisphenol 'X' family has no PPARγ$_2$ agonist activity in the PPARγ$_2$-CALUX® assay, but they are PPARγ$_2$ antagonists. Increasing concentrations of BPA, BPF and BPS were tested in both agonist (Panels A-C) and antagonist modes in the presence of 50 nM of RGZ (Panels D-F). Each test was performed both in the presence (dotted lines) or absence (plain lines) of S9 metabolizing system. All agonistic activities measured were below the LOQ of the test system, indicating no effect. All antagonist activities measured occurred above 30 µM, indicating weak antagonism.

Family	Substance	Agonist	Antagonist
BPx	BPA		+
	BPF		+
	BPS		+
Halogenated BPA	TCBPA	+	+
	TBBPA	+	+
Diglycidyl-ether	BADGE		+
	BFDGE		+

CONCLUSIONS

- The PPARγ$_2$-CALUX® assay is able to give consistent results, similar to those found in the literature → **fit-for-purpose**
- No proof of principle could be found to detect the effect of metabolism on PPARγ assay results. *In silico* modeling indicated that phase I metabolism is not likely to play a critical role in the PPARγ$_2$-mediated activity of chemicals → **application of the S9-protocol not recommend in routine testing for PPARγ**
- Antagonistic activities detected for all BPX compounds tested; only TCBPA and TBBA presented PPARγ$_2$ agonistic activities
- In general, agonist efficacy is low as compared to the rosiglitazone reference (e.g. TBT) → **data interpretation is difficult**
- Obesogens could activate PPARγ through mechanisms **not involving its direct activation**, which may be more important than expected; these are not detected by this assay.
- **More understanding** of the field is necessary before considering this assay as a first priority for routine screening in an in *vitro* test battery.