

DETERMINATION OF REP VALUES FOR THE CALUX® BIOASSAY AND COMPARISON TO THE WHO TEF VALUES

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Introduction

Previously the CALUX® (Chemically-Activated LUciferase eXpression) assay has been used to screen biological samples for contamination by dioxin-like compounds¹. A validation study has also been reported that examined ash, soil and exhaust gas samples by both gas chromatography/mass spectrometry (GC/MS) and the CALUX® assay in a double-blinded study². These studies indicated that the CALUX® assay can be used to detect the presence of dioxin like compounds and is predictive of GC/MS results. In order to better understand the relationship between GC/MS results and in order to better characterize the responsiveness of the CALUX® bioassay, relative potency (REP) values were determined for each of the active polychlorinated dibenzodioxin and polychlorinated dibenzofuran (PCDD/PCDF) congeners and selected coplanar polychlorinated biphenyls (PCB). Generally the results were consistent with the consensus toxic equivalency factors (TEFs) reported by the World Health Organization³, but there were examples that diverged from the WHO TEF values. These differences seem to be related to the fact that the WHO TEF values incorporate uptake and metabolism whereas these processes do not appear to have a significant impact on the responsiveness of the CALUX® bioassay.

Materials and Methods

PCB standards were purchased from AccuStandard, Inc. (New Haven, CT). Dioxin and dibenzofurans standards were purchased from Wellington Laboratories, (Guelph, Ontario, Canada).

CALUX® Assay: XDS has developed a cell line (mouse hepatoma H1L1) that was stably transfected with a vector that contains the gene for firefly luciferase under transactivational control of the aryl hydrocarbon receptor⁴. Serial dilutions of the compounds of interest were prepared in dimethyl sulfoxide. Prior to dosing the cells, the DMSO solutions were suspended in cell culture medium. This medium was then used to expose monolayers of the H1L1 cell line grown in 96 well culture plates. In addition to the samples, a standard curve of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (1287, 322, 161, 80.5, 40.2, 20.1, 10.1, 5.0, 2.5, 1.2 and 0.6 parts per trillion (ppt) TCDD) was assayed on each plate for comparison. The plates were incubated for 20 hours in a humidified CO₂ incubator. Following incubation, the medium was removed and induction of luciferase activity was quantified using the luciferase assay kit from Promega (Madison, WI).

Data analysis: The response for each concentration of each compound was analyzed at least three times. Data for the dose response series were fit to a sigmoid curve described by the Hill Equation using least squares best fit modeling. The values for the maximal response and concentrations associated with 20-80% of the maximal response (EC₂₀₋₈₀) were determined from the derived Hill

Equation for each compound. The maximal response for each of the compounds was compared to the maximal response for TCDD using a two tailed student's t-test with $\alpha = 0.05$.

Results and Discussion

In order to determine whether the REP values that were obtained could be used accurately to assess biological risk it is necessary to make sure that the efficacy of the compound being tested and the slope of the dose response curve for the compound are equivalent to the corresponding values for 2,3,7,8-TCDD⁵.

Determination of Efficacy for Tested Compounds: Efficacy is the maximal response for the compound expressed as a percentage of the maximal response for TCDD. The maximal response for the compound being tested was compared to TCDD using a two-tailed student t-test. Using $\alpha = 0.05$, eight out of the 17 active PCDD/PCDF congeners were found to have maximal responses that differed from TCDD (see Table 1). This seemed to be largely due to the relatively small variation in the maximal response for TCDD. Of the seventeen congeners, none differed by more than 25% from the maximal response that was measured for TCDD which suggests that this should not be a significant concern in regards to using the CALUX[®] bioassay for risk assessment for PCDD/PCDF congeners. In contrast the maximal response (efficacy) for the PCB compounds that were tested were predominantly lower than the maximal response for TCDD. Five out of six were statistically different from TCDD at the $\alpha = 0.05$ level. Three PCBs (77, 114 and 156) exhibited maximal responses that were approximately one half the maximal response for TCDD. At higher concentrations of these PCB the response in the CALUX[®] assay would be expected to plateau at a lower luciferase expression than could be obtained by exposing the cells to TCDD. This could lead to an underestimation of the contribution of the PCB to the TEQ. As such it would be preferable to always quantify the contribution of PCB to TEQ at a point less than one half the maximal response for TCDD. By diluting unknown samples until the response in the CALUX[®] assay is less than half the maximal response for the TCDD standard curve, the determination of total TEQ from PCB in the sample should avoid inaccuracies that would be associated with the lower efficacy of these compounds.

Evaluation of the Effect of the Slope of the Dose Response Curve on REP Values: If the slope of the dose response curve for the tested compound and the slope of the dose response curve for TCDD differ, then the REP value obtained for the tested compound will be different depending on which part of the curve is used to determine the ratio between the two compounds. The REP values for effective concentrations from 20% of maximal response to 80% of maximal response (EC₂₀₋₈₀) were determined and the range for the REP values were reported. This provided a means to assess the potential affect of the difference in slope of the two dose response curves on the REP value and eventually the use of the bioassay for determining biological risk for the tested compound.

Comparison of REP Values to WHO TEF Values: The consensus TEF values reported by the WHO are stated to be order of magnitude estimates³. These estimates are based on *in vivo* and *in vitro* experiments and thus incorporate processes such as uptake, tissue distribution, metabolism and receptor binding and activation. For cell based assays, uptake and tissue distribution are not relevant to the response to compounds. Metabolism could potentially have an impact on the responsiveness of the cells, but we have not seen any indication of significant metabolism of the test compounds during the exposure period (data not shown). As expected, it appears that in the CALUX[®] assay the REP values are predominantly dependent on receptor binding and activation. Our results reveal an excellent correlation between the REP values and the WHO TEF values.

Only one PCDD/PCDF congener, (OCDF), differs by more than an order of magnitude. There does seem to be a pattern in that the more highly chlorinated PCDD/PCDF congeners (hepta and octachlorinated) have REP values that are higher than the TEF values reported by the WHO. This could be related to the fact that these compounds have lower absorption rates than are seen for PCDD/PCDF congeners in general⁶. The lower absorption rates for these compounds could have the affect of decreasing the TEF values. The cell based assay does not incorporate absorption in the REP value and thus would be expected to be higher than a toxicity equivalency factor that does incorporate absorption.

Table 1. Results from the comparison of the dose response curves for the active dibenzodioxins, dibenzofurans and selected coplanar polychlorinated biphenyls to 2,3,7,8-tetrachlorodibenzo-p-dioxin. REP values are reported as ng/ml.

Compound	WHO-TEF ³	CALUX REP, based on EC ₅₀	REP Range, (EC ₂₀ to EC ₈₀)	B _{max} = TCDD B _{max} 2 tailed, a = 0.05	Efficacy, % of TCDD B _{max}
TCDD	1	1.00 +/- 0.01			
12378-PeCDD	1	0.73 +/- 0.10	0.44 to 1.02	no	114%
123478-HxCDD	0.1	0.075 +/- 0.014	0.034 to 0.137	yes	105%
123678-HxCDD	0.1	0.098 +/- 0.017	0.043 to 0.183	no	123%
123789-HxCDD	0.1	0.061 +/- 0.012	0.028 to 0.114	no	119%
1234678-HpCDD	0.01	0.031 +/- 0.008	0.015 to 0.058	yes	101%
OCDD	0.0001	0.00034 +/- 0.00008	0.00025 to 0.00049	yes	87%
2378-TCDF	0.1	0.067 +/- 0.010	0.040 to 0.104	no	93%
12378-PeCDF	0.05	0.14 +/- 0.04	0.14 to 0.15	yes	87%
23478-PeCDF	0.5	0.58 +/- 0.08	0.37 to 0.78	no	106%
123478-HxCDF	0.1	0.13 +/- 0.02	0.07 to 0.20	no	109%
123678-HxCDF	0.1	0.14 +/- 0.03	0.10 to 0.19	yes	101%
123789-HxCDF	0.1	0.11 +/- 0.02	0.05 to 0.18	no	112%
234678-HxCDF	0.1	0.31 +/- 0.06	0.31 to 0.31	yes	91%
1234678-HpCDF	0.01	0.024 +/- 0.007	0.019 to 0.031	yes	96%
1234789-HpCDF	0.01	0.044 +/- 0.010	0.032 to 0.059	yes	107%
OCDF	0.0001	0.0016 +/- 0.0005	0.0003 to 0.0058	no	125%
PCB 77	0.0005	0.0014 +/- 0.0004	0.0012 to 0.0017	no	53%
PCB 81	0.0001	0.0045 +/- 0.0012	0.0022 to 0.0085	no	89%
PCB 114	0.0005	0.00014 +/- 0.00002	0.00014 to 0.00017	no	45%
PCB 126	0.1	0.038 +/- 0.007	0.037 to 0.042	yes	94%
PCB 156	0.0005	0.00014 +/- 0.00002	0.00013 to 0.00019	no	53%
PCB 169	0.01	0.0011 +/- 0.0003	0.0007 to 0.0017	no	69%

Comparison of the REP values for PCB also suggests a pattern, but in the case of the PCB the lower chlorinated compounds seem to have REP values that are higher than would be expected based on the WHO TEF values. In particular PCB 77 and PCB 81 have REP values that are 2.8 and 45 times greater than the corresponding WHO TEF values. The REP values for the rest of the PCBs tend to be somewhat lower than would be predicted based on the WHO TEF values. The higher than expected values for PCB 77 and PCB 81 could be associated with their metabolism. In whole animals these compounds are metabolized⁷, which would result in a lower TEF value. In the CALUX[®] assay these compounds are not significantly metabolized during the exposure period (data not shown). The lack of metabolism by the CALUX[®] cells could contribute to the higher than expected REP values.

Conclusions

The experiments that are described in this report have helped to better characterize the responsiveness of the CALUX[®] assay to compounds that bind to and activate the Aryl hydrocarbon receptor. The REP values that have been determined for the CALUX[®] assay indicate that the responsiveness of the CALUX assay corresponds well with the TEF values reported for these compounds by the WHO. There are differences, which is to be expected considering that a cell based bioassay does not incorporate all of the mechanisms that contribute to the overall activity and toxicity of a compound. The hepta and octa chlorinated dioxins/furans and the tetrachlorinated biphenyls have higher REP values than would be expected based on WHO TEF values. This could lead to over estimation of the TEQ for samples that are contaminated primarily by these compounds. However, over estimation by the CALUX[®] assay is not a significant concern. The CALUX[®] assay is intended to be a screening assay that can be used to identify samples that need to be analyzed by more time consuming and expensive chemical analysis methods (GC/MS). In general it is better for a screening assay to provide a high estimate as false positives are more acceptable than false negatives for a screening assay.

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