

Antagonistic effects of methyl-mercury and PCB153 on PC12 cells after a combined and simultaneous exposure

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Received 5 September 2005; accepted 19 April 2006

Abstract

The study of interactions for those substances which tend to accumulate in food and affect the nervous system appears to be a fundamental point to characterize the combined exposure *in vitro*. In this study we included two food contaminants which are known neurotoxicants: methyl-mercury (Me-Hg) and the *ortho*-substituted PCB 153.

PC12 cells were treated with Me-Hg (range $1e-7$, $2e-6$ M) and PCB153 (range $1e-5$, $4e-4$ M) in single and combined synchronous experiments and a mathematical model was set up according to the Loewe additivity criterion to evaluate the level of interaction between toxicants, using viability as end-point.

At some concentrations (Me-Hg $5e-7$ M and PCB153 $1e-4$ and $2e-4$ M; Me-Hg $1e-6$ M and PCB153 $5e-5$ M; Me-Hg $1e-7$ M and PCB153 $4e-4$ M), a statistically significant antagonist effect was observed. No interaction was observed for other combinations.

The analysis of other toxicological parameters known to be modified in single exposure experiments (TBARS and intra-cellular dopamine) confirmed the viability results.

The results of our work represent a starting point to generate novel information on the interactions between PCB153 and Me-Hg *in vitro*, as well as a new relevant experimental and mathematical approach useful to investigate the effects of different toxicant mixtures. © 2006 Elsevier Ltd. All rights reserved.

Keywords: Methyl-mercury; PCB153; PC12; Neurotoxicity; Combined exposure

Abbreviations: DA, dopamine; DMSO, dimethylsulphoxide; HPLC, high performance liquid chromatography; Me-Hg, methyl-mercury; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PCB, polychlorinated biphenyls; SD, standard deviation; TBARS, thio-barbituric acid reactive substances.

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1. Introduction

Environmental toxicants can enter the very low stages of the food chain as a result of man's action. Even if their use is banned or regulated in order to minimise the exposure in the environment, their residues are persistent pollutants and food intake is the major exposure route both for humans and most wildlife, leading to bioaccumulation and biomagnification (Mackay and Fraser, 2000; Kelly et al., 2004; Luoma and Rainbow, 2005).

Many of these toxicants cause varying degrees of neurological damage. The effects on the developing neurological system are of particular concern since adults and children/

neonates have different degrees of susceptibility to such neurotoxicants.

The neurotoxic food contaminants included in this study are methyl-mercury (Me-Hg) and the *ortho*-substituted PCB 153.

Chronic exposure to low doses of mercurial organic compounds is a worldwide health concern: it could be pathogenetically relevant as co-factor in several neurodegenerative diseases and its neurotoxic effect is well known (ATSDR, 1999). In fact, excessive ingestion from a diet high in fish can lead to a dangerous toxicant burden with clinical or sub-clinical implications (Gray et al., 2005; Jarrell et al., 2005; Senn et al., 2005). Me-Hg has a high affinity for SH-groups and therefore forms complexes with various thiol-compounds. Perturbation of intra-cellular Ca^{2+} and induction of oxidative stress are also major mechanisms responsible for Me-Hg toxicity (Humphrey et al., 2000).

PCB153 belongs to PCBs' family, a class of 209 congeners of toxic and biopersistent synthesis compounds. These compounds are used in numerous industrial products such as diluents, hydraulic fluids, dielectric fluids for transformers and capacitors, heat transfer fluids, etc. Because of their lipophilic nature and resistance toward biotransformation, PCBs accumulate in the food chain (Muhlebach et al., 1991). As exposure assessment has improved, it has emerged that even levels commonly experienced by the general population can cause adverse effects. Cognitive impairments, decreased verbal abilities and reduced psychomotor development have been associated to prenatal exposure to PCBs (Muhlebach et al., 1991; Li et al., 1994; ATSDR, 2000; Carta et al., 2003). PCB153 non-planar congener is among the PCBs that appear most prevalently in the environment, in human serum (Grandjean et al., 2004) and in the mammalian tissues at the highest concentration (Mahaffey, 2000). Moreover, it has been shown to have estrogenic effects (Castoldi et al., 2001).

Because Me-Hg and PCBs are environmental pollutants and tend to accumulate in the same type of food, particularly fish, a concomitant exposure to Me-Hg and PCBs has been observed. It is therefore relevant to study the neurotoxic effects of co-exposures both in vivo and in vitro (Rice, 2000; Newland and Paletz, 2000; Grandjean et al., 2001).

The traditional experimental approach to study toxicity and to assess risk is based on a single substance characterization, either in vivo or in vitro. Certainly, such basic research is essential for the understanding of the compound significance and mechanism of action. Although the effects of mixtures of neurotoxicants are of more practical significance, there have been a few attempts to analyse the interactions between combinations of compounds in vitro, which have provided evidence for potential synergies that may enhance toxicity (Svendgaard et al., 1997).

In the literature many different approaches related to mixtures have been reported (Feron and Groten, 2002). A very simple method to define a non-interaction value

for a combination experiment is the Bliss independence criterion (Greco et al., 1992; Suehnel, 1998), while with the Loewe additivity criterion, introduced by Loewe (1953) and developed by Berenbaum (1985), it is possible to define a non-interaction surface in a n -dimensional space for a combination experiment (Suehnel, 1998). When the combination effects deviate from the zero-interaction surface and the total effect is higher than the expected one a Loewe/Bliss synergism is defined. On the contrary, if the total effect is lower than the expected one, an antagonistic interaction is present. Although the two general methods have been compared with appositely studied software (Dressler et al., 1999), no agreement on which of the two models is more appropriate exists. However, independence criterion has been preferred in special field of toxicology, as combined irradiation (Groten et al., 2001). A current application of Loewe additivity approach is based on the isobolographic method (Gessner, 1995; Schoen, 1996). Notably, the non-interaction curve has been calculated for simple mathematical functions especially for binary mixtures (Suehnel, 1998; Dressler et al., 1999) and more complex mathematical models have been adopted (Gennings, 1996; Bae et al., 2001; Charles et al., 2002).

In the present study we address the problem of the simultaneous exposure to different neurotoxicants in an established dopaminergic in vitro model, namely in the PC12 cell line. PC12 cells were treated in single and combined exposure experiments with the two selected compounds (Me-Hg and PCB153) and viability data were used to set up a mathematical model to evaluate interactions according to the Loewe additivity approach. In addition, to check the validity of the method, other toxicological parameters known to be modified in single exposure experiments by the selected neurotoxicants (levels of lipid peroxidation by means of TBARS and levels of intra-cellular dopamine), were tested for the statistically significant combinations of concentrations extrapolated from the mathematical model.

2. Materials and methods

2.1. Chemicals and reagents

For cell cultures, RPMI 1640, horse serum and foetal bovine serum (heat inactivated) were purchased from Gibco BRL, Life Technologies Ltd. (Paisley, Scotland); penicillin (5000 units/ml) and streptomycin (5000 µg/ml) from ICN Biochemicals, Inc. (Irvine, CA). Flasks and 96-well plates were obtained from Costar, Corning Inc. (Corning, NY).

For cell treatment and MTT assay, hydrochloric acid (HCl), anhydrous dimethylsulfoxide (DMSO), Triton X-100, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), PCB153 were from Sigma-Aldrich Chemie, anhydrous isopropanol from Fluka Chemie AG (Buchs, Switzerland) and methylmercury (II) hydroxide from Alfa Aesar (Karlsruhe, Germany).

For TBARS assay, 1,1,3,3-tetraethoxypropane (Malondialdehyde) was obtained from Fluka Chemie (Buchs, CH-9471), *N*-buthanol from Aldrich (Steinheim, Germany and Milwaukee, USA, respectively). Thiobarbituric acid was from Sigma Chemical Co. (St. Louis, MO, USA). For catecholamine analysis, all HPLC chemicals (of analytical grade) were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell line and treatments

Rat pheochromocytoma PC12 cells (American Type Culture Collection, ATCC, Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% horse serum, 5% foetal bovine serum and 25 units/ml of penicillin + 25 µg/ml of streptomycin.

For MTT assay, exponentially growing cells were collected, centrifuged, suspended at 10^5 cells/ml in fresh medium and 100 µl were dispensed in each well of a 96-well culture plate. For Trypan Blue Exclusion test, TBARS assay and intra-cellular dopamine analysis, cells were seeded in a 25 cm² flasks (10^6 cells/flask). Cells were allowed to grow for 24 h in a humidified atmosphere of 5% CO₂, 95% air at 37 °C before the treatment, then 10 µl and 500 µl of a 10-fold concentrated solution of the toxicants prepared in free-sera medium was added to each well or flask, respectively. DMSO was used as solvent for PCB153 at a final concentration not toxic for cells (lower than 0.4%, very far to the toxic concentrations we experimentally found – EC10 between 5% and 10%, data not shown). Me-Hg stock solution was prepared in water. In single exposure experiments the following ranges of concentrations were tested: 0, 1e–7, 5e–7, 1e–6, 2e–6, 4e–6 M (Me-Hg) and 0, 1e–5, 5e–5, 1e–4, 2e–4, 4e–4 M (PCB153). In co-exposure experiments, toxicants were added simultaneously to the medium and all the possible combinations of the concentrations listed above were tested. Treated cells were allowed to grow for 24 h.

2.3. Cell viability assay

The MTT assay was carried out to evaluate cell viability (Mossman, 1983). The method used in this study is described in Goldoni et al. (2003). Samples were cultured in eight replicates at each concentration tested and experiments were performed in duplicate. Untreated controls and blanks were incubated in the same plates and under the same conditions.

2.4. Trypan blue exclusion test

To evaluate cell membrane damage characteristic of necrosis and late apoptosis, cells were harvested and an aliquot of the cell suspension was mixed with an equal volume of 0.4% Trypan blue in phosphate-buffered saline (PBS). Cells were scored at the phase contrast microscope using a Neubauer improved counting chamber. Samples were cultured in triplicate and experiments were performed three times. Statistical analysis was performed with the Student *t*-test, comparing controls and exposed cells.

2.5. Detection of TBARS

Cellular TBARS were measured according to the method of Jentsch et al. (1996) and Vettori et al. (2005), adapted for cells. TBARS concentrations were normalized for the number of viable cells as assessed by the Trypan Blue exclusion test. Samples were cultured in triplicate and experiments were performed three times.

2.6. High performance liquid chromatography (HPLC) intra-cellular dopamine analysis

At the end of the incubation period, cell samples were collected according to Alinovi et al. (1996) and analysed in HPLC (Vaarmann et al., 2002). Intra-cellular DA concentrations were normalized for the number of viable cells as assessed by the Trypan Blue exclusion test.

2.7. Mathematical model

The following paragraphs describe the model that has been applied to study the combination of the two selected toxic substances in vitro (PCB153 and Me-Hg). Notably, it could be extended to the *n*-dimensional space (*n* toxic substances).

2.7.1. Introducing the model

The Loewe dose-additivity law defines zero interaction by the following equation (Suehnel, 1998):

$$\frac{d_1}{D_1} + \frac{d_2}{D_2} = 1 \quad (1)$$

where D_1 and D_2 are the doses of the single toxic substances that produce an effect, E , and d_1 , d_2 are the doses of the same substances that produce the same effect in a combination experiment. If the sum is <1, the interaction is synergistic, while if it is >1, it is antagonist. On the basis of Eq. (1), it is possible to apply this model only if the mathematical functions describing a determined effect follow the principle:

$$\exists D_1, D_2 : d_1, d_2 : E(D_1) = E(D_2) = E(d_1, d_2) \quad (2)$$

While the Bliss independence criterion can be applied only to fractional effects ($0 < E < 1$), the Loewe additivity law does not necessarily require this normalization (Suehnel, 1998).

In this study, the fractional cell viability was determined using the MTT assay (see Section 2.3). In addition, to fit the experimental data deriving from the single exposure experiments to PCB153 and Me-Hg (dose–response curves), the Hill function (3) has been used, according to Goldoni et al. (2003):

$$V_i = V_{0,i} \left(1 - \frac{x^{n_i}}{EC50^{n_i} + x^{n_i}} \right) \quad (3)$$

where *i* identifies the *i*th toxic substance, $V_{0,i}$ is cell viability in the absence of the toxicant, n_i the co-operativity index and x the concentration of the toxicant. With the Levenberg–Marquart algorithm (Origin 7.0, Originlab corporate, Northampton, MA, USA) it is possible to fit the experimental data with this type of Hill function, maintaining V_0 , EC50 and n as variable parameters. Origin 7.0 calculates also the SD associated to these parameters.

Suehnel (1998) showed the equation of the non-interaction surface for the Hill function in the simple case where the co-operativity index is the same for both the toxicants considered. Since, in general, n varies among substances, in particular for PCB153 and Me-Hg, in the present study a more complex equation was calculated.

2.7.2. Assumptions of the model

- (1) $\lim_{\text{Me-Hg} \rightarrow 0} V(\text{Me-Hg}) = \lim_{\text{PCB153} \rightarrow 0} V(\text{PCB153}) = V_0 = 1$, for the non-interaction curve. In the absence of toxic substances, in fact, differences in V_0 values of the two pure curves should be due only to an experimental variability and not to a real difference. Therefore, being V_0 kept variable in the fitting, both values found for Me-Hg and PCB153 were normalized to 1, and the highest value of SD associated with the two V_0 calculated parameters was selected as SD for both substances.
- (2) $\lim_{\text{Me-Hg} \rightarrow +\infty} V(\text{Me-Hg}) = \lim_{\text{PCB153} \rightarrow +\infty} V(\text{PCB153}) = 0$, as showed by the function used to fit the experimental data.

2.7.3. Building the non-interaction surface

Using the Loewe additivity law and performing some mathematical passages, it is possible to demonstrate that, having different values of EC50 and co-operativity indexes (n), we can not obtain an explicit surface equation $V = V(d_1, d_2)$, as in simple cases (Suehnel, 1998). If $d_1 = [\text{Me-Hg}]$, $d_2 = [\text{PCB153}]$ in the combination experiments, an implicit equation describes the non-interaction surface:

$$d_1(\text{EC50})_2 Y^{\frac{1}{n_2}} + d_2(\text{EC50})_1 Y^{\frac{1}{n_1}} = (\text{EC50})_1 (\text{EC50})_2 Y^{\frac{n_1+n_2}{n_1 n_2}} \quad (4)$$

where 1 and 2 refer to Me-Hg and PCB153, respectively and $Y = \frac{V_0 - V}{V}$, being d_1 and d_2 the experimental doses chosen for the combination experiment and EC50 and n the different values extrapolated by the non-linear fitting on the pure curves. Changing d_1 and d_2 , it is possible to calculate Y values with some specifically created programs (for example

with MATLAB language), or solving graphically the equation with Y as variable. Alternatively, the program FUNCTIONS (www.numericalmathematics.com) can automatically find the zero of the function $f(Y) = 0$.

Finally, we can find V as $V = \frac{V_0}{1+Y}$ and build a 3-dimensional surface in space.

2.7.4. The maximum and minimum slope curves

Whereas Suehnel (1998) and Dressler et al. (1999) calculated the non-interaction surface, they did not take into account of the intrinsic error of the pure curves, and they did not propose a statistical method to compare the experimental points of the combination experiment with the derived interaction 0 curve.

Fig. 1 shows a simulation of the Hill function. On the basis of SD of the parameters kept variable in the fitting of the pure curves, it is possible to find the minimum and maximum slope curve:

$$(1) \text{ Maximum } V_{\text{MAX}} = (V_0 + \delta V_0) \times \left(1 - \frac{x^{n+\delta n}}{(EC50 + \delta EC50)^{n+\delta n} + x^{n+\delta n}} \right), \quad \forall x < x_{\text{max}} \quad (5)$$

$$V_{\text{MAX}} = (V_0 + \delta V_0) \times \left(1 - \frac{x^{n-\delta n}}{(EC50 + \delta EC50)^{n-\delta n} + x^{n-\delta n}} \right), \quad \forall x > x_{\text{max}} \quad (6)$$

$$(2) \text{ Minimum } V_{\text{MIN}} = (V_0 - \delta V_0) \times \left(1 - \frac{x^{n-\delta n}}{(EC50 - \delta EC50)^{n-\delta n} + x^{n-\delta n}} \right), \quad \forall x < x_{\text{min}} \quad (7)$$

$$V_{\text{MIN}} = (V_0 - \delta V_0) \times \left(1 - \frac{x^{n+\delta n}}{(EC50 - \delta EC50)^{n+\delta n} + x^{n+\delta n}} \right), \quad \forall x > x_{\text{min}} \quad (8)$$

where x_{max} and x_{min} are in general the cross points of the two curves of maximum and minimum, and δ represents the SD values on the parameters. These points vary both on the basis of the fitting curve and on the amplitude of the SD of the parameters and the minimum and maximum non-interaction surfaces can be extrapolated using Eq. (4) where $V = V_{\text{MAX}}$ and V_{MIN} for both Me-Hg and PCB153.

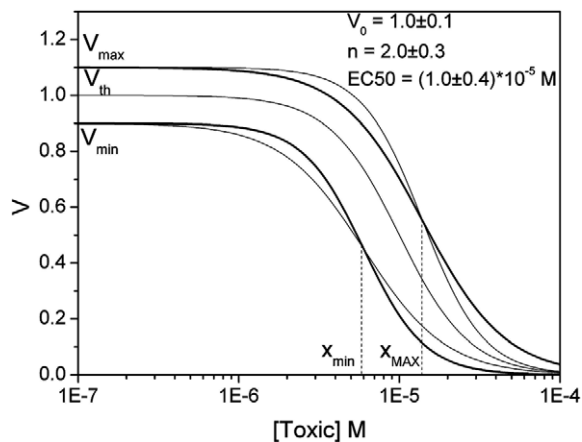


Fig. 1. Simulation of the Hill function with the maximum and minimum slope curves. x_{max} is the intercept between the functions of Eq. (5) (—) and (6) (—), while x_{min} between the functions of Eq. (7) (—) and (8) (—). The parameters of the simulation curve (with SD) are also reported.

In conclusion, every theoretical point (V_{th}) on the non-interaction curve has a confidence interval, in general asymmetric, given by ($V_{\text{th,MIN}}, V_{\text{th,MAX}}$). Therefore, in first approximation we can consider as SD of the point $\text{Max} \{V_{\text{th}} - V_{\text{th,MIN}}, V_{\text{th,MAX}} - V_{\text{th}}\}$. Finally, considering $V_{\text{th}} \pm \text{SD}$ and $V_{\text{exp}} \pm \text{SD}_{\text{exp}}$, where the last is the experimental value of V found in the combination experiment (the number of experimental data used to build every theoretical point is $n1 + n2$, where $n1$ and $n2$ are the number of experimental points used for the pure curves for Me-Hg and PCB153, respectively), with an independent t -student test it is possible to assess the significance of the difference between theoretical and experimental viability values. A value of $p < 0.05$ was considered significant.

3. Results

Fig. 2 shows the pure viability curves of Me-Hg and PCB153 together with their corresponding maximum and minimum curves. The two compounds showed a different degree of toxicity. EC50 values vary from 1.2×10^{-6} M for Me-Hg to 2.2×10^{-4} M for PCB153. Moreover, both curves showed co-operativity values (n) significantly higher than 1. Dose–response curves obtained with Me-Hg dissolved in 0.4% of DMSO and with Me-Hg dissolved in serum-free medium were overlapping (data not shown).

Combining the effects of Me-Hg and PCB153 according to Eq. (4) and using the pure curve equations and their maximum and minimum slope curves, we obtained the 3-D graph showed in Fig. 3. In addition, the experimental viability was measured at different combined concentrations of Me-Hg and PCB153. Table 1 reports the comparison between experimental and theoretical non-interaction points. As shown in Fig. 3 and in Table 1, at some combinations of concentrations (Me-Hg 5×10^{-7} M and PCB153 1×10^{-4} and 2×10^{-4} M; Me-Hg 1×10^{-6} M and PCB153 5×10^{-5} M), a statistically significant antagonist effect was observed. A similar effect was observed for co-exposure to lower doses of Me-Hg (1×10^{-7} M) and to doses of PCB153 higher than EC50 (4×10^{-4} M).

In order to test if the found combinations of concentration have the same competitive effect also on other

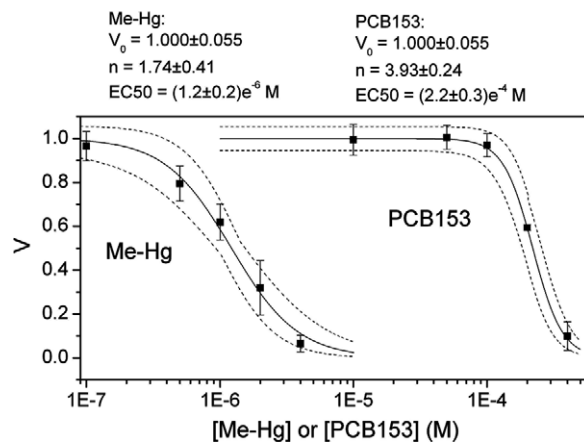


Fig. 2. Dose–response curves reporting variation in PC12 cells viability after single exposure to Me-Hg and PCB153. This figure shows also the maximum and the minimum slope curves (dotted lines), the experimental points with SD used and the parameters of the fitting functions.

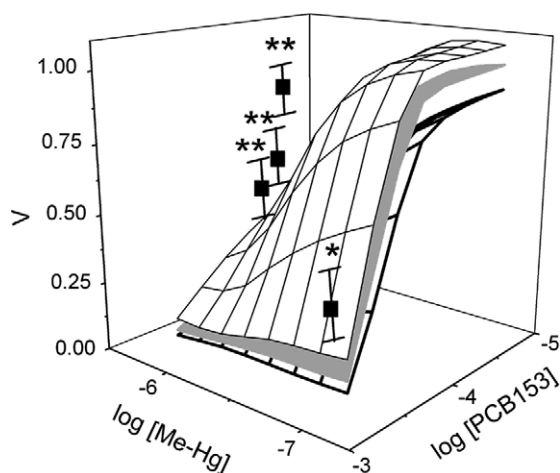


Fig. 3. Comparison between some viability experimental data points and the zero interaction surface. The 3D-surfaces were calculated using the best fit curves, the minimum and maximum slope curves reported in Fig. 2 by Eq. (4) (* $p < 0.05$; ** $p < 0.01$).

Table 1

Viability of PC12 cells at different concentrations of Me-Hg and PCB153 obtained in combination experiments

[Me-Hg], M	[PCB153], M	V_{th}	Range	V_{exp}	SD_{exp}
1e-7	5e-5	0.96	0.85–1.04	0.96	0.11
1e-7	1e-4	0.88	0.74–0.99	0.93	0.13
1e-7	2e-4	0.51	0.36–0.68	0.55	0.15
1e-7	4e-4	0.08	0.04–0.15	0.25*	0.12
5e-7	5e-5	0.72	0.58–0.87	0.88	0.13
5e-7	1e-4	0.59	0.46–0.74	0.93**	0.07
5e-7	2e-4	0.31	0.24–0.42	0.59**	0.08
1e-6	5e-5	0.47	0.39–0.59	0.73**	0.08
2e-6	5e-5	0.23	0.13–0.35	0.30	0.12
2e-6	1e-4	0.18	0.10–0.29	0.15	0.06

V_{th} (range) values represent the viability calculated with the mathematical model; V_{exp} and SD_{exp} were the experimental observed data points; * $p < 0.05$ and ** $p < 0.01$, p -values performing a t -student test (two tailed) between the experimental and theoretical data points. The combinations not reported were not significantly different. We considered as SD for the V_{th} value the highest difference between V_{th} and the extremes of the range.

end-points different from viability, the levels of lipid peroxidation (TBARS, marker of oxidative stress) and variation in DA intra-cellular levels, have been measured in PC12 cells.

The levels of TBARS, indicative of lipid peroxidation, were dose-dependently increased in single dose experiments, for both the tested compounds (data not shown). DMSO (0.4%) did not significantly modify the TBARS increase induced by Me-Hg dissolved in serum-free medium (data not shown). Fig. 4 summarizes the obtained results after single and combined exposure to the selected compounds, using some combination of concentrations derived from the mathematical approach. A significant increase in the levels of lipid peroxidation was evident after single exposure to both the compounds as compared to controls ($p < 0.01$ for Me-Hg and PCB153). Notably, lipid peroxidation resulted lower in co-exposed samples, as compared to single compound exposure, confirming the competitive effect of Me-Hg and PCB153 in this cellular model.

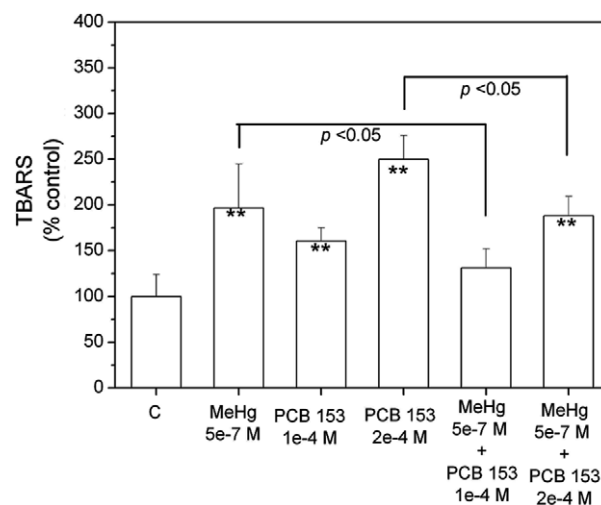


Fig. 4. TBARS levels in single and combined experiments with Me-Hg and PCB153. To compare the levels of TBARS at different experimental conditions, one-way ANOVA followed by Tuckey post hoc tests was used (** $p < 0.01$).

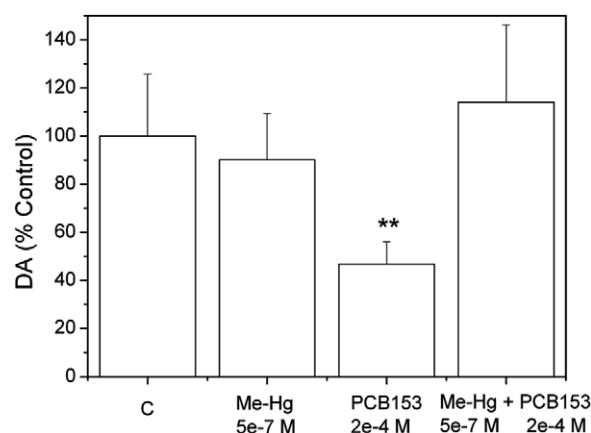


Fig. 5. Dopamine intra-cellular levels in single and combined experiments with Me-Hg and PCB153. To compare the levels of dopamine at different experimental conditions, one-way ANOVA followed by Tuckey post hoc tests was used. The combination of Me-Hg and PCB153 1e-4 M is not reported, because this concentration of PCB did not reduce significantly the dopamine levels (** $p < 0.01$).

As for TBARS, also intra-cellular dopamine content, was dose-dependently decreased after exposure to Me-Hg and PCB153 (data not shown). Using the combination of doses extrapolated from the mathematical approach the intra-cellular dopamine levels reported in Fig. 5 were obtained. Comparing the levels obtained in single exposure experiments (5e-7 M Me-Hg and 2e-4 M PCB153) with the ones deriving from the simultaneous exposure, a competitive effect was evident.

4. Discussion

In our study we have addressed the complex problem of simultaneous exposure to two different neurotoxicants using an in vitro approach.

PC12 cells were treated with the selected compounds (Me-Hg and PCB153) in single and combined experiments. Data were analysed using a mathematical model set up according to the Loewe additivity approach.

We have found that, at some combination of concentrations, Me-Hg and PCB153 showed antagonistic effects, while no significant interactions were observed for most combination concentrations. In addition, synergistic effects were never observed in the range of concentrations considered. The validity of the method was confirmed by the analysis of other toxicological parameters known to be modified in single exposure experiments to the selected neurotoxicants (levels of lipid peroxidation and levels of intra-cellular dopamine).

The mathematical approach used in this study derives from a previously reported model based on the Loewe additivity criterion (Suehnel, 1998) and reinforces the idea that the identification of a function describing the pure dose–response curve is an essential point to assess the toxicity of a chemical. In the present study, the Hill function has been applied to obtain the pure dose–response curves representing variation in viability of PC12 cells treated with Me-Hg and PCB153 (Goldoni et al., 2003). The model proposed here presents several advantages. First, it is applicable to every combination of mathematical functions with two or more toxicants. Second, since it takes into account the maximum and minimum dose–response curves (obtained using SDs calculated from the function fitted using experimental data), it allows the statistical comparison between the expected theoretical values and the experimental data obtained from the co-exposure experiments. However, the use of interaction-zero surfaces is not widely accepted, in particular where the single dose response curves are very steep and Loewe additivity gives larger combination effects than Bliss independence (Dressler et al., 1999). Bliss independence criterion, on the other hand, is based on statistical calculation and does not take into account of the shape of the dose–response curves of the single toxic compounds.

In this study the model has been applied to analyse viability data (MTT assay) obtained treating PC12 cells with pure and combined Me-Hg and PCB153. Moreover, at those concentrations where an antagonist effect was observed, we reinforced the obtained results pointing the attention on two parameters known to be affected by single exposure to the selected neurotoxicants: intra-cellular concentrations of an oxidative stress marker (TBARS, indicative of lipid peroxidation), and intra-cellular dopamine levels.

Oxidative stress has been implicated in neurotoxic damage of different cellular types associated with Me-Hg (Sanfeliu et al., 2001; Shanker et al., 2004, 2005) and elevated levels of TBARS were reported (Yee and Choi, 1996). Moreover, oxidative stress induced by Aroclor 1254 has been considered a cause of dopaminergic cell injury (Lee and Opanashuk, 2004) and has been involved among cell death causes of rat cerebellar granule cells after exposure

to the same mixture (Mariussen et al., 2002). Finally, a PCB-mediated release of dopamine has been observed on PC12 cells (Angus and Contreras, 1996), and PCB153 has been associated to apoptosis of neuronal cell cultures (Sanchez-Alonso et al., 2003).

The interaction of PCBs and Me-Hg both in vivo and in vitro is controversial. Widholm et al. (2004) concluded that combined exposure to PCBs (Aroclor 1254) and Me-Hg does not exacerbate the PCB- or Me-Hg-induced impairments on spatial alternation tasks in rats. On the other hand, Roegge et al. (2004) presented probable synergistic effects of developmental exposure to PCBs (Aroclor 1254) associated with Me-Hg on three motor tasks involving cerebellar functions in rats. Moreover, Bemis and Seegal (1999) showed that PCBs (Aroclor 1254/1260) and Me-Hg act synergistically to reduce rat brain dopamine content in vitro, while synergistic, additive and antagonistic effects, depending on the 2,2'-dichlorobiphenyl concentrations, were observed at the level of $[Ca^{2+}]$ regulation in rat cerebellar granule cells (Bemis and Seegal, 2000). Recently, Fischer et al. (2004) showed that PCBs (PCB153) and Me-Hg could interact during a critical stage of neonatal brain development to enhance developmental neurotoxic effects, even if at relative high concentrations of PCB153 (0.5 mg/kg).

Notably, Aroclor is a mixture of different PCBs and the molecular basis of its synergistic effect with Me-Hg could be due to the interaction between the different PCB congeners and the metal. Also the use of an in vitro approach could be useless to investigate on this complex interaction. Moreover, a competitive or simply additive effect of PCB153 and Me-Hg observed in vitro does not necessarily imply that a synergistic interaction could not exist in vivo, being the exposure routes and the modality of interaction between toxicants and neurons (direct or indirect) important aspects to be considered.

In this study we report an antagonistic interaction between some combinations of concentrations of PCB153 and Me-Hg. Antagonistic effects are reported in literature after a combined exposure of cancerous cellular models to different chemotherapies (Peters et al., 2000). Some examples of this kind of interaction are also present in the environmental toxicology field (Chen and Lu, 2002; Katsifis et al., 1998).

In vitro, different mechanisms could account for an antagonistic interaction: (a) if the toxic substances show different affinities towards the same intra-cellular binding sites, one of the two selected compound could not be able to explicit completely its toxicity. Competitive binding is a well-known biochemical methodology to test the affinity of substrates to proteins and receptors; (b) if one of the two toxicants has higher affinity towards the binding sites, even at relatively low concentrations, it could exert only a slight effect on cells. Nevertheless, the rapid kinetic of binding between the compound and the binding sites would be able to induce defence and detoxification mechanisms, lowering the second toxicant effect. This mechanism could be partic-

ularly relevant in the case of compounds with different degree of toxicity; (c) a direct chemical interaction between the toxicants could exist. Indeed, the antagonism could be due to all the above mentioned mechanisms.

On the basis of both intra-cellular dopamine levels and viability data, it seems possible that Me-Hg, the most toxic substance, has an effect at relatively low concentration ($5e-7$ M) on PCB153 toxicity, pointing to the second mechanism as the one that could explain the observed results. Nevertheless, the combined exposure to PCB 153 and Me-Hg decreased the content of cellular peroxidated lipids, as compared to the single Me-Hg exposure, suggesting a significant role also of the first proposed mechanism.

Since a direct chemical interaction between Me-Hg and PCBs has never been seen in literature, the third listed mechanism needs deeper chemical investigations. Further studies are in progress with asynchronous exposures to Me-Hg and PCB153 to better characterize these mechanisms of antagonistic toxicity.

In conclusion, the definition of synergistic, antagonistic or additive effects appears to be a fundamental point to characterize the combined exposure to different toxicants in vitro, in particular for those substances which accumulates in food. In the present study we propose a very flexible mathematical model to be used in vitro to assess the level of interaction between toxicants assessing the zero interaction surface. The results of our work represent a starting point to generate novel information on the interactions between PCB153 and Me-Hg in vitro, and it proposes a new relevant experimental approach that could be used to investigate the effects of other types of toxicant mixtures.

Conflict of interest statement

All the authors declare that they have no competing interests.

Acknowledgement

Supported by EC (Contract number: FOOD-CT-2003-506143).

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