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Hydrolyzing activities of phenyl valerate sensitive to organophosphorus compounds paraoxon and mipafox in human neuroblastoma SH-SY5Y cells

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ABSTRACT

The molecular targets of best known neurotoxic effects associated to acute exposure to organophosphorus compounds (OPs) are serine esterases located in the nervous system, although there are other less known neurotoxic adverse effects associated with chronic exposure to OPs whose toxicity targets are still not identified. In this work we studied sensitivity to the non-neuropathic OP paraoxon and to the neuropathic OP mipafox of phenyl valerate esterases (PVases) in intact and lysed human neuroblastoma SH-SY5Y cells. The main objective was to discriminate different unknown pools of esterases that might be potential targets of chronic effects from those esterases already known and recognized as targets to these acute neurotoxicity effects. Two components of PVases of different sensitivities were discriminated for paraoxon in both intact and lysed cells; while the two components inhibitable by mipafox were found only for intact cells. A completely resistant component to paraoxon of around 30% was found in both intact and lysed cells; while a component of slightly lower amplitude (around 20%) completely resistant to mipafox was also found for both preparations (intact and lysed cells). The comparison of the results between the intact cells and the lysed cells suggests that the plasma membrane could act as a barrier that reduced the bioavailability of mipafox to PVases. This would imply that the discrimination of the different esterases should be made in lysed cells. However, those studies which aim to determine the physiological role of these esterases should be necessarily conducted in intact cultured cells.

1. Introduction

The main toxic effects of the organophosphorus compounds (OPs) compounds are due to the non-reversible phosphorylation of esterases of the central nervous system. Different known toxicity targets exist, as do some cases in which these targets are unknown. Acetylcholinesterase (AChE) and neuropathy target esterase (NTE) are the most known and are the more widely studied target esterases of OPs toxicity. Inhibition of AChE by phosphorylation triggers the known acute cholinergic effects responsible for the insecticide activity of OPs, which cause symptoms of acute intoxication (miosis, vomiting, weak muscles, fasciculations, flaccidity, difficulty in breathing, heart arrhythmias) (Jokanović and Kosanović, 2010). The inhibition of NTE by OPs and their subsequent aging triggers a late polyneuropathy, whose symptoms include flaccidity, ataxia in lower limbs and paralysis (Jokanović and Kosanović, 2010).

Certain exposures to OPs can trigger other neurotoxic effects, like that known as intermediate syndrome, or can cause OPs-induced delayed polyneuropathy. Intermediate syndrome is characterized by difficulties in breathing and weak neck and limb muscles and, as previously mentioned, the mechanism underlying this effect remains unknown (Costa, 2006). Establishing the doses of the NTE-inhibiting compounds that are not able to induce its aging (e.g., some non-neuropathic OPs or phenylmethysulfonyl fluoride) has been described as protecting against OPs which age the enzyme and trigger late neuropathy (Costa, 2006). However, when these compounds are administered after the agent that induces neuropathy, the neuropathy symptoms are exacerbated (Jokanovic et al., 2002). The target that promotes this exacerbation of the delayed polyneuropathy still remains unknown to date, although it can be stated that it is not NTE because in the sequence in which the agents interacting with it are administered, NTE's active center would be occupied by neuropathic agents when protecting agents are administered; moreover, the promoting phenomenon also appears with neuropathy that takes place without NTE inhibition, like those induced by physical aggression pressing a nerve or by exposure to either 2,5-hexanodione or bromophenyl urea (Jokanovic et al., 2002).

Abbreviations: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; CbEs, carboxylesterase enzymes; NTE, neuropathy target esterase; OP, organophosphorus compounds; PBS, phosphate buffer saline; PV, phenyl valerate; PVases, phenyl valerate esterases

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It has been suspected that chronic exposure to OPs could induce neuropsychiatric disorders without cholinergic symptoms appearing. This would rule out high levels of AChE inhibition as a toxicity target of these efforts. The commonest symptoms are cognitive deficits (damaged memory, concentration and learning), anxiety, depression, psychosis, chronic tiredness, unstable posture, rigid face muscles or dystonia (Jokanović and Kosanović, 2010; Baltazar et al., 2014; Terry et al., 2012). The toxicity targets of these effects caused by chronic exposure are still unknown.

Carboxylesterase enzymes (CbEs) are a particulat type of esterases that catalyze the hydrolysis reactions of carboxylic esters, with many of them existing in different tissues and living organisms. Several classifications of esterases exist, one of them uses as criteria their interaction with OPs. According to this criterion, Aldridge (1989) established three types of esterases as: A-esterases, that are esterases that hydrolyze both carboxylesterases and OPs; B-esterases are those esterases that hydrolyze carboxylesterases (both endogenous and exogenous) and are irreversibly inhibited by OPs (i.e., the potential target CbEs of toxicity like AChE or NTE). The third type of esterases, namely C-esterases, has no type of interaction with OPs.

Phenyl valerate esterases (PVases) are a group of CbEs (B-esterases) that can be studied by phenyl valerate (PV) carboxyl ester hydrolysis. In this group we find NTE, which can be differentiated from other PVases given their paraoxon-resistance activity and their sensitivity to mipafox. Cholinesterases are included in the group of B-esterases (CbEs), where serine hydrolases are responsible for degrading the acetylcholine neurotransmitter. Two different types of cholinesterases exist in vertebrates, namely AChE and butyrylcholinesterase (BuChE), which are differentiated by their substrate specificity. AChE and BuChE are also potential targets of the toxicity of OPs, and inhibition of AChE triggers the above-cited cholinergic effects. However, the inhibition of BuChE not only does not cause apparent adverse effects, but also protects from cholinergic effects acting as a bioscavenger by removing one OP molecule from the medium before it reaches and inhibits AChE. Thus, administering exogenous BuChE has been considered a therapy and prophylaxis when faced with exposure by OPs (Reed et al., 2017; Lushchekina et al., 2018).

The study of CbEs inhibition kinetics by different OPs in raw tissues allows the different groups of CbEs present in this tissue to be discriminated (Estevez and Vilanova, 2009; Mangas et al., 2016), and can potentially identify and discriminate the presence of CbEs, different to AChE, BuChE and NTE, with an unknown physiological functions and, thus, potential targets of the toxicity of OPs. Therefore, by using this methodology in the animal model normally used to study the neurotoxicity of OPs, adult chicken (Organization of Economic Cooperation and Development, 1995a,b), it has been possible to discriminate the following enzymatic components of PVase activity: EI, EII and EIII in the soluble peripheral nervous system fraction; Es1, Es2 and Es3 in serum; Eα, Eβ and Eγ in the soluble brain fraction; and EPα, EPβ, EPγ and EPδ in brain membranes. These components are clearly distinguished in the sensitivity and reversibility of the interaction that takes place with several OPs, such as paraoxon, mipafox, among others (Estévez et al., 2004, 2010, 2011, 2012; Mangas et al., 2011, 2012a,b, 2014a). Semi-preparative chromatographic fractioning of Eα activity (Mangas et al., 2014b) and its subsequent mass spectroscopy analysis has confirmed the presence of BuChE (Mangas et al., 2017a), although it cannot be ruled out that other target proteins of OPS are found among the 258 proteins that have been identified to date.

Competition experiments between substrates have suggested that a relationship exists between PVases sensitive to OPs and hydrolyzing acetylcholine enzymes in chicken brain (Benabent et al., 2014). BuChE's capacity to hydrolyze PV has also been demonstrated in humans, which entails having to include cholinesterases among the potential targets of toxicity of OPs (Mangas et al., 2017b).

Thus by taking all this into account we conclude that, a series of adverse effects induced by OPs whose toxicity targets have still not been identified exists and that is might be assumed that such targets would be esterases of the nervous system with no specific substrates. Thus, it would thus be necessary to identify them based on the sensitivity to different OPs agents. The hen is the accepted animal model for study OP neuropathy and its promotion, but it would not necessarily be useful to study the toxic effects that stem from chronic exposure to OPs. So it would be worthwhile shifting to human cell models. Preliminary data suggest that human neuroblastoma SH-SY5Y cells express PVases, and that at least the most well-known targets of toxicity (AChE and NTE) are inhibited by OPs and are comparable with chicken brain (Sogorb et al., 2010). This work attempts to discriminate different fractions of PVases based on their sensitivity to paraoxon and mipafox, which are two widely used OPs as models of non-neuropathic and neuropathic compounds, respectively.

2. Materials and methods

2.1. OPs and substrates

Mipafox was purchased from Lark Enterprises (Webster, USA) with purity higher than 99%. A concentrated solution was prepared in 10 mM Tris/10 mM Citrate buffer /1 mM EDTA pH 6.0, which was kept at 4 °C. Paraoxon was obtained from Sigma-Aldrich Química S.A. (Madrid, Spain) with purity higher than 90%. The stock solution was prepared at 10 mM in dry acetone. PV was supplied from Lark Enterprises (Webster, USA) and a 30 mg/ml stock solution was prepared in dimethylformamide.

2.2. Cell cultures

The human neuroblastoma SH-SY5Y cell line was employed in all the tests, which was obtained from the European Collection of Authenticated Cell Cultures (ECACC; https://www.phe-culturecollections.org.uk/collections/ecacc.aspx; catalogue number 94,030,304). The cell culture medium was prepared following the supplier's instructions and contained Dulbecco's Modified Eagle Medium with 4500 mg glucose/ml supplemented with 44.5% of nutrients HAMF12 with $NaHCO_3$, 10% heat inactivated fetal bovine serum and 1% of a prepared antibiotics mix (5000 units of penicillin/ml, 5 mg of streptomycin/ml and 10 mg of neomycin/ml). Cells were seeded in 60-ml flasks that contained a total of 10 ml culture medium and were incubated at 37 °C in a 5% CO_2 atmosphere until cell confluence at 90–95% was reached. When this confluence was reached and immediately prior to starting each experiment adhered cells were removed from plates by trysinization and were resuspended at appropriate density and in a suitable medium according to the purpose of the experiment. No nerve growth factor or retinoic acid were included in the cell culture media and therefore the cells were growth in undifferentiated state.

2.3. Cell lysis

For cell lysis purposes, cell cultures were incubated and resuspended in 10 mM Tris/1 mM EDTA buffer pH 7.4 in an ice bath for 35 min. This treatment proved to be efficient to cause total cell lysis (Fig. S1, Supplementary Material). Conversely, when the incubation was performed with phosphate buffer saline (PBS) no significant lysis was observed (Fig. S2, Supplementary Material). No statistically significant differences were recorded among the PVases activities with the samples incubated in an ice bath for 35 min in either PBS or in 10 mM Tis/1 mM EDTA buffer pH 7.4.

2.4. Protein quantification

The DC assay (Bio-Rad Laboratories) was used in order to quantify the protein present in cell suspensions. The supplier's indications were

followed using bovine serum albumin as the standard.

2.5. PVase activity assay

Experiments in intact and lysed cell suspensions were performed simultaneously. To determine the enzymatic hydrolysis of the PV substrate after exposure to OPs, 100 µl of intact or lysed cell suspension (100 µl of buffer if blanks were used) were treated with 20 µl of the corresponding inhibitor (or buffer if blanks) for 30 min at 37 °C. After this time, 100 µl of the PV substrate (1 mg/ml) were added and the mixture was incubated at 37 °C for another 30-minute period. The enzymatic reaction was stopped by adding 100 µl of a 2% SDS/4 mg/ml aminoantipyrine solution. Finally, 50 µl of a 2% (w/v) potassium cyanide solution in water were added. The formation of a reddish compound generated as the result of reducing the phenol-aminoantipyrine complex was determined by recording absorbance at 510 nm. The quantity of phenol released by PVases was determined by comparing the absorbance of each sample with the absorbance of a phenol calibrated line.

2.6. Mathematical models

The data obtained in the experiments were fitted to different mathematical models using the Sigma Plot software (version 8.0). The program uses a Marquardt-Levenberg algorithm that seeks the values of the parameters to be determined, in such way that the sum of the squares of the differences between the real value and the value predetermined by the equation is minimal. The employed mathematical models were developed by Estevez and Vilanova (2009), by assuming irreversible inhibition. To inhibit several enzymatic components, the residual activity after the inhibition time would be the sum of the residual activities of each enzymatic component. The general formula is shown below:

$$E = E_1 + E_2 + \ldots + E_R$$

Depending on how the enzymatic systems behavior, the mathematical models include a different number of components. The employed models were:

- A component that is sensitive to inhibition: $E = E_0 \cdot e^{(-k_1' \cdot I)}$
- A sensitive component and a resistant one: $E = E_{1_0} \cdot e^{(-k_1' \cdot I)} + E_{R_0}$
- Two sensitive components: $E = E_{1_0} \cdot e^{(-k_1' \cdot I)} + E_{2_0} \cdot e^{(-k_2' \cdot I)}$
- Two sensitive components and a resistant one: $E = E_{1_0} \cdot e^{(-k_1' \cdot I)} + E_{2_0} \cdot e^{(-k_2' \cdot I)} + E_{R_0}$

In these models, E is defined as the active free enzyme, E_{1o} is the amplitude of the most sensitive enzymatic component, E_{2o} is the amplitude of the least sensitive enzymatic component, E_{R0} is the amplitude of the enzymatic component that is absolutely resistant to OP, k_1' is the first-order inhibition constant of the first enzymatic component, k_2' is the first-order inhibition constant of the second enzymatic component and I is the employed inhibitor concentration.

In a system with three enzymatic components, two sensitive ones (E_1 and E_2) and a resistant one (E_R) at the inhibitor concentration (I), the behavior of the different inhibition curves is described by this equation:

$$\%\,\text{Activity} = 100 \cdot \frac{E}{E_0} = 100 \cdot \frac{E_{1_0} \cdot e^{(-k_1' \cdot I)} + E_{2_0} \cdot e^{(-k_2' \cdot I)} + E_R}{E_0}$$
$$= 100 \cdot \frac{E_{1_0} \cdot e^{(-k_1' \cdot I)}}{E_0} + 100 \cdot \frac{E_{2_0} \cdot e^{(-k_2' \cdot I)}}{E_0} + 100 \cdot \frac{E_R}{E_0}$$
$$= E_1 \cdot e^{(-k_1' \cdot I)} + E_2 \cdot e^{(-k_2' \cdot I)} + E_R$$

where E_{1o} and E_{2o} represent the proportion (amplitude) of the sensitive components of enzymes E_1 and E_2, E_o is the control of the initial activity (with no inhibitor), E is the activity for an inhibitor concentration I, k_1' and k_2' represent the second-order inhibition constants (the product of the first-order inhibition constant and the inhibition time, which was always constant, 30 min) for each enzymatic component expressed and are expressed in μM^{-1}, and E_R is the proportion of the resistant component (the residual activity resistant to the high used inhibitor concentrations).

The Sigma Plot mathematical fits were done following some restrictions required for the solution to be of biological significance and plausibility. These restrictions were: k_1', k_2', E_1, E_2, $E_R > 0$; and $(E_1 + E_2 + \ldots + E_R) = 100$.

The concentration at which 50% of the component "n" was inhibited after a 30-minute of exposure (IC_{50}) can be calculated with this equation:

$$I_{50}n = \frac{\ln 2}{k_n'}$$

Whether more complex mathematical models would present a statistically significant improvement compared to simpler models was verified using an F test performed by the Sigma Plot 8 software. The selected model in each case was the simplest one that best fitted the experimental data.

3. Results

3.1. Hydrolyzing PV activities in human neuroblastoma SH-SY5Y cells

Intact and lysed cells displayed PVase activity. The total mean PVase activity of intact cells was 4.40 ± 1.29 nmol phenol/30 min. µg protein for seven independent cultures, while the total mean PVase activity of the lysed cells (from the same cultures but as intact cells) was 5.06 ± 3.38 nmol phenol/30 min. µg protein for the same seven independent cultures. Differences in activity were not statistically significant. The PVase activities of both preparations were sensitive to paraoxon and mipafox.

3.2. Paraoxon inhibition of hydrolyzing PV activities in human neuroblastoma SH-SY5Y cells

3.2.1. Intact cells

In three independent experiments run to test inhibition by paraoxon in intact cells within a range of concentrations from 10 to 360 µM, the biologically plausible mathematical model that best fitted with the experimental data was that with two sensitive components (with no independent term). Table 1 shows the mathematical parameters obtained in the fits of the three independent experiments. The second component (K_2 between 0.0024 and 0.0040 μM^{-1}) possessed an IC_{50}

Table 1
The mathematical parameters that fitted the inhibition of the PVase activities of human neuroblastoma SH-SY5Y cells by paraoxon. It is displayed individual records found in each independent experiment. Fig. 1 plots the results corresponding to the experiment with paraoxon concentration between 2.5 and 1500 µM.

		Component 1			Component 2			
Cells	Paraoxon (µM)	E_1 (%)	K_1 $(\mu M)^{-1}$	IC_{50} (µM)	E_2 (%)	K_2 $(\mu M)^{-1}$	IC_{50} (µM)	E_r (%)
Intact	10–360	14	0.185	3.7	86	0.0040	172	–
		32	0.089	7.8	68	0.0024	279	–
		39	0.193	6.1	61	0.0035	200	–
	2.5–1500	34	0.441	1.6	40	0.0023	303	26
Lysed	10–360	11	0.193	3.6	89	0.0029	239	–
		20	0.500	1.4	80	0.0033	210	–
	2.5–1500	24	0.496	1.4	44	0.0017	397	32

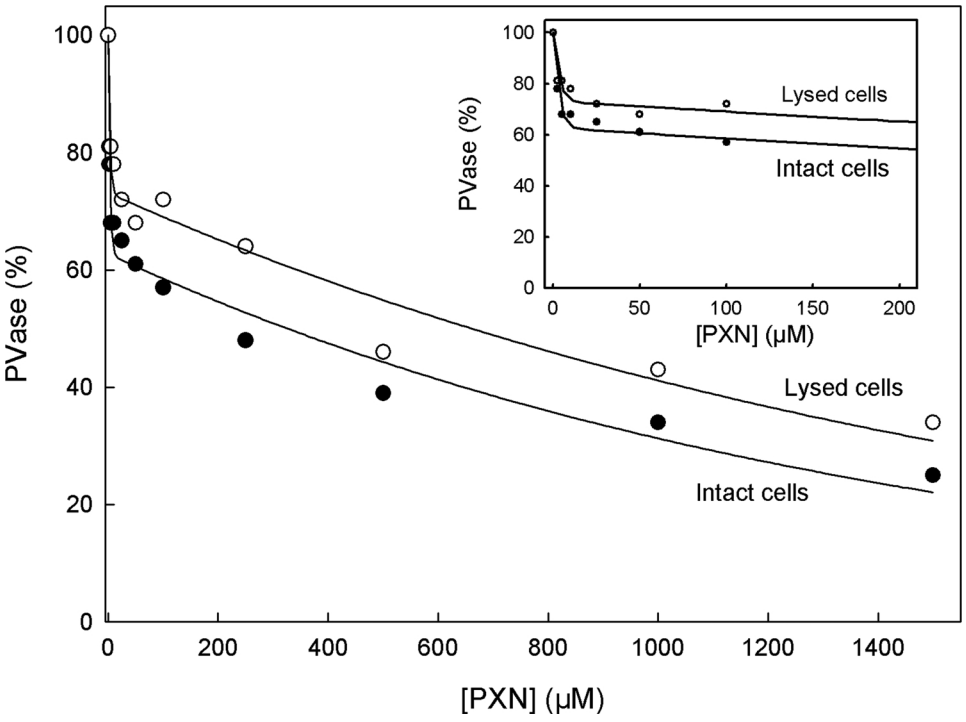

Fig. 1. Inhibition by paraoxon of the PVase activity of intact and lysed human neuroblastoma SH-SY-5Y cells. Both cell preparations were exposed for 30 min at 37 °C to different paraoxon concentrations and residual PVase activity was determined as described in Section 2.5. In both cases the best and simplest mathematical model that fitted the obtained experimental data was that with the two sensitive components plus one resistant component with the parameters shown in Table 1. The PVase activity of both preparations in the absence of paraoxon is indicated in Section 3.1.

between 172 and 279 μM, so it was not completely inhibited by the highest tested paraoxon concentration (360 μM). Because of that a fourth experiment was run, in which paraoxon concentrations between 2.5 and 1500 μM were tested. In this case the simplest and most biologically plausible mathematical model that best fitted the experimental results was that with two sensitive components and one resistant to paraoxon (Table 1, Fig. 1). There was a large difference in sensitivity between these two components because IC_{50} of the component 2 was 189 times higher than the IC_{50} of the first one, while the amplitudes of both components were quite similar (between 34 and 40%) and slightly higher than the resistant term of 26% amplitude (Table 1).

3.2.2. Lysed cells

In the two independent experiments in which paraoxon concentrations between 10 and 360 μM were tested, the simplest and most biologically plausible mathematical model was that with two sensitive components and no resistant component (Table 1). However, as in the case of the intact cells the most resistant component (IC_{50} mean of both independent experiments 225 μM) was not totally inhibited by the highest paraoxon concentration employed in these experiments (again 360 μM). Moreover, the plot of residual PVase versus paraoxon concentration of these plots (data not shown) suggested that the lowest concentration employed in the experiment (10 μM) had already totally inhibited the most sensitive component (IC_{50} mean of two independent experiments of 2.5 μM, Table 1). These two facts brought the necessity to perform another experiment with a wider range of paraoxon concentration in order the obtain data that might define in a better way the two apparent paraoxon sensitive components.

When a range of concentrations between 2.5 and 1500 μM was used the mathematical model that fitted to the experimental data considered two paraoxon-sensitive components plus a totally resistant term. Amplitudes and sensitivities of these three distinguished components were in the same order of magnitude than those deduced in similar conditions using a preparation of intact cells (Table 1).

3.3. Inhibition by mipafox of the hydrolyzing PV activities in human neuroblastoma SH-SY5Y cells

3.3.1. Intact cells

For the four independent experiments run, the simplest and most biologically plausible mathematical model that explained the experimental observations was that with two sensitive components and one resistant component to mipafox. Table 2 shows the mathematical parameters that resulted from the cited fit, and Fig. 2 represents one of the three PVase activity experiments. The component with the widest amplitude was around 8 times more sensitive to mipafox than the most resistant one, while the amplitude of the totally resistant component was between 10 and 20% (Table 2).

3.3.2. Lysed cells

For the four independent experiments run, the simplest and most biologically plausible mathematical model that best fitted with the experimental results was that with one totally resistant component plus one sensitive component. Table 2 shows the mathematical parameters that resulted from the cited fit, while Fig. 2 plots residual PVase activity

Table 2
The mathematical parameters that fitted the inhibition of the PVase activities of human neuroblastoma SH-SY5Y cells by mipafox. It is displayed individual records found in each independent experiment. Fig. 2 plots the results corresponding to one of the four independent experiments.

	Component 1			Component 2			
Cells	E_1 (%)	K_1 $(\mu M)^{-1}$	IC_{50} (μM)	E_2 (%)	K_2 $(\mu M)^{-1}$	IC_{50} (μM)	E_r (%)
Intact	57	0.064	11	29	0.0048	145	14
	50	0.043	16	38	0.0027	262	13
	40	0.155	41	41	0.0062	112	19
	56	0.157	31	31	0.0054	129	13
Lysed	89	0.075	9.3	–	–	–	11
	79	0.061	11	–	–	–	21
	79	0.113	6.1	–	–	–	21
	75	0.110	6.3	–	–	–	25

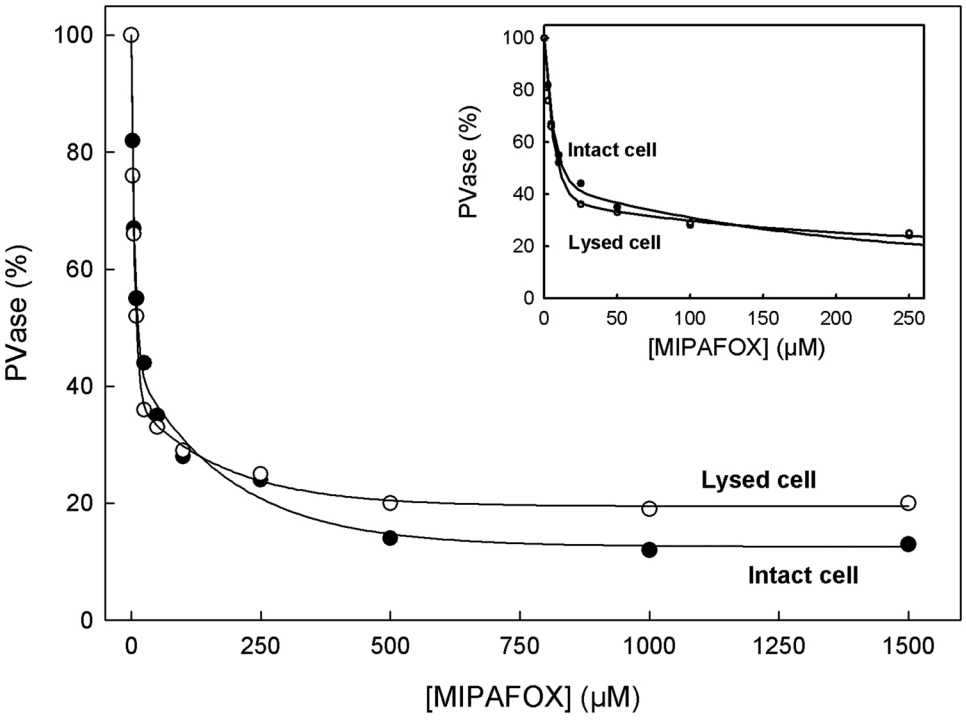

Fig. 2. Inhibition by mipafox of the PVase activity of intact and lysed human neuroblastoma SH-SY-5Y cells. Both cell preparations were exposed for 30 min at 37 °C to different mipafox concentrations and residual PVase activity was determined as described in the Materials and Methods. The best mathematical model that fitted the obtained experimental data in intact cells was that with the two sensitive components, plus one resistant component with the parameters shown in Table 2. The best mathematical model that fitted the obtained experimental data in lysed cells was that with one sensitive components plus one resistant component with the parameters shown in Table 2. The PVase activity of both preparations in the absence of mipafox is indicated in Section 3.1.

versus mipafox concentration for one of the four independent experiments. The amplitude of the totally resistant component was quite similar to amplitude reported for such component in intact cells (Table 1); while the sensitivity (IC_{50}) was in the same order of magnitude (Table 2).

The possibility of a behavior similar to the reported for intact cells (based on two sensitive components plus a totally resistant one) was biologically implausible, since the IC_{50} of the most resistant component displayed an IC_{50} in the same order of magnitude than reported for intact cells but the most sensitive component would have been 400 orders of magnitude more sensitive than the most resistant (data not shown).

4. Discussion

4.1. PV hydrolyzing activities in human neuroblastoma SH-SY5Y cells

Ehrich (1995) also demonstrated that neuroblastoma SH-SY5Y cells possessed not only AChE, which was inhibited by a wide range of OPs, but also NTE (a particular type of PVase), which was only inhibited by neuropathic OPs, including mipafox, which we also used herein. These same authors also demonstrated that the PVases and AChE of human neuroblastoma SH-SY5Y and mouse neuroblastoma NB41A3 were inhibited by 11 different OPs, but PVases in human cells were more resistant than AChE for 9 of these 11 OPs, and for all 11 in mice cells (Ehrich and Correll, 1998).More recently, our group showed that NTE expressed by human neuroblastoma SH-SY5Y cells responded similarly to that expressed in chicken brain (the most usual animal model employed to test in vivo delayed neuropathy induced by OPs) in inhibition, reactivation and aging terms. This allowed this model to be used as an alternative in vitro method for animal experiments to evaluate OPs' capacity to induce neuropathy (Sogorb et al., 2010). Hence, this is not the first work to describe PVases and AChE activities that can be inhibited by OPs in human neuroblastoma SH-SY5Y cells but, as far as we are aware, it is the first attempt to dissect the different components of esterases sensitive to OPs based on their sensitivity to distinct OPs.

4.2. Hydrolyzing activities of phenyl valerate sensitive to paraoxon

Regardless of statistical and mathematical considerations, the behavior of the intact cell system is biologically consistent with a resistant component with IC_{50} of at least 200 μM or higher and with an amplitude of about 70% (the amplitude of component A_2 in the model with no independent term or the sum of the amplitudes of components A_2 and the resistant component in the model that considers an absolutely resistant component); plus a more sensitive component whose amplitude is approximately 30% with an IC_{50} of between 1.6 and 7.8 μM (Table 1). This model is also consistent with that published previously by our research group, where the same cell model included PVases activities that were sensitive to paraoxon with an IC_{50} of 6 μM and an amplitude of 15%, while the remaining 85% was considerably more resistant (IC_{50} = 407 μM) (Sogorb et al., 2010).

In the experiments run with lysed cells, we found a similar situation to that of intact cells. The mathematical model that best explained the experimental results included a component whose amplitude was 24% with similar sensitivity to the most sensitive component found in intact cells; while the second component of around 44% of amplitude was (as in the case of intact cells) at least 300 times more resistant than the first one (Table 1). The amplitudes of the totally paraoxon-resistant terms were barely distinguishable.

We can see that the IC_{50} of the more resistant component in intact cells was slightly lower than that of the lysed cells. These differences, whether could be biologically relevant, might be explained whether we hypostatize that this pool of paraoxon-sensitive PVases might be located in the plasmatic membrane with active center outside the cell and therefore the bioavailability in intact cells might be slightly higher than in lysed cells because the experiments were run with whole homogenates (without a physical separation of particulate and cytoplasmic material).

4.3. Hydrolyzing activities of phenyl valerate sensitive to mipafox

The experimental results indicated that the PVase activities of intact and lysed SH-SY5Y cells presented an absolutely resistant component to this neuropathic OP with amplitude between 15% and 25% (Table 2).

In both systems (intact and lysed cells) a sensitive component was detected with an IC_{50} of between 6 and 41 μM (Table 2). In intact cells, a second sensitive component appeared with an IC_{50} of 162 μM (mean of the four independent experiments) that was not detected in lysed cells (Table 2). This second component resistant to mipafox which appeared in intact cells could not be detected in lysed cells. It might be interpreted considering that in intact cells the bioavailability of mipafox to PVases could be lower than in lysed cells whether such enzymes were in whatever intracellular compartment because mipafox would have to cross plasmatic membranes by diffusion before to reach the target centers and; indeed, mipafox is an much polar OP than paraoxon and therefore the difficulties to pass through lipid bilayers would theoretically be higher than for paraoxon.

4.4. Conclusions

Human neuroblastoma SH-SY5Y cells express pools of PVases distinguishable on the basis of their sensitivity to the OPs paraoxon and mipafox (Tables 1 and 2; Figs. 1 and 2). Therefore, it is very plausible that other PVase populations might be screened and discriminated using other OPs using similar approaches to those already employed in our group using chicken brain tissues (Estevez et al., 2004; Estevez and Vilanova, 2009; 2010, 2011, 2012; Mangas et al., 2011, 2012a,b, 2014a,b). It suggests that these cells could be used as a good model to discriminate and identify esterases in the human nervous system which remain unknown to date and could be targets of toxicity of other less characterized effects as; e.g., the neuropsychiatric disorders attributed to chronic exposure to low concentrations of OPs; promotion of neuropathy (with its potential repercussions on preventing neurodegenerative diseases, even whose etiology is not necessarily related with exposure to OPs); etc.

The results displayed in Tables 1 and 2 show certain differences between preparation of intact and lysed cells. Taking into consideration that there were not statistically significant differences in the total PVases in both preparations, it seems that there were differences in bioavailability of OPs to active center of PVases. These differences might be addressed either to polarity of each specific OP, that might determine higher or lower to pass through plasmatic membrane by diffusion; or to cellular location of the active center of the esterase (by example pointing towards extracellular milieu or within an intracellular membrane as endoplasmic reticulum).

Our data suggest that experimental studies conducted to discriminate the different esterases sensitive to several OPs should be done in lysed cells rather than in intact cells, even though the studies that aim to determine the physiological role of these esterases should necessarily be done in intact cultured cells.

To discriminate the new potential targets of OPs toxicity according on an inhibition kinetics basis, it would be necessary to use a series of broader OPs and employ more complex mathematical models, like those developed by Estevez and Vilanova (2009), which include the possibility of analyzing certain effects like simultaneous and concurrent inhibition with the substrate, reactivation, etc.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tox.2018.07.016.

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