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Cytotoxicity of Phenolic Compounds on *Dicentrarchus labrax* Erythrocytes

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The use of *in vitro* model for toxicological studies is specially appropriate to analyze the impact of molecules on well defined targets, or to study the mecanisms involved in cell toxicology. In this purpose, we previously demonstrated that marine fish erythrocytes can be suitably developed as a good cell model to investigate the effects of metallic salts on membrane integrity or on detoxification enzyme activities (Roche et al. 1991; Gwozdzinski et al. 1992; Roche and Bogé 1993). In contrast to mammalian red blood cells, fish erythrocytes are nucleated and well provided with subcellular organelles. In fish, these cells are directly exposed to plasma xenobiotics absorbed through gills or via the digestive tract, and/or biotransformed in liver. We applied this cell model to phenolic compounds with a special interest in the characterization of the relationship between their structure and membrane or metabolic integrity. These structure-effects relationships offer the possibility to predict the toxicity of xenobiotics on the basis of some physico-chemical properties taken as reference (Könemann 1981; Kavlock et al. 1991).

Phenol and its derivatives are classically used in the preparation of plastic, synthetic fibers, detergents, pesticides, stainers, additives, as in petroleum refining, etc. Some of them have physiological or pharmacological activity, whereas others are major atmospheric or aquatic pollutants. These compounds are highly absorbed through biological membranes and are concentrated in organisms where their biodegradability is generally elevated. In humans, some cause hemolysis or methemoglobinization. At the cellular level, they affect notably mitochondrial respiration, membrane potential or synaptic transmission, which explains their acute neurotoxicity (Kaila 1982; Bradbury et al 1989). Due to this high biological reactivity, their appearance in water causes severe toxicity to fauna and their presence in consumption waters is routinely checked. The classification of these compounds has been done according to their structure and to some physicochemical properties: partitioning coefficient and pKa (Leo et al. 1971; Schultz 1987). The biological parameters which have been considered, are related to cell viability, as to enzyme activities involved in cell protection towards oxygen active derivatives. The viability of the erythrocytes incubated during 24 hr in the

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presence of phenol or its derivatives has been evaluated on the basis of the percentage of hemolysis and on the decrease of cell ATP content. Enzymes concerned in cell defense were superoxide dismutase (SOD), catalase, peroxidase and glutathion peroxidase (GSH-Px). The initial concentration of phenols was 1 mM. When some noticeable toxicity was detected, lower concentrations were assayed until the disappearance of this effect.

MATERIALS AND METHODS

Sea bass (*Dicentrarchus labrax;* 200-300 g) were obtained from DEVA-Sud, experimental Farm of IFREMER, Palavas les flots, France. Whole blood of 5 fishes was withdrawn by caudal puncture and pooled. Red blood cells were washed, at 4°C with an isotonic solution of sodium chloride (NaCl 170 mM) and incubated in a marine salt solution (Sigma ref S-9148, 12.3 $g.L^{-1}$). This cell suspension medium had a sodium concentration and an osmolality equivalent to sea bass plasma (370 $mosm.L^{-1}$; Na^+ 154.6 mM; K^+ 3.2 mM; Mg^{2+} 17.8 mM;Ca^{2+} 3.4mM; Cl^- 181 mM; HCO_3 0.81 mM; SO_4^{2-} 9.15 mM; D-glucose 5.55 mM, HEPES 1 mM; pH 7.45). Each batch of erythrocytes was exposed for 24 hr at 20°C in 20 mL of a solution containing 1 µM, 10 µM, 100 µM or 1 mM of phenol compounds. Each chemical was tested on at least five batches of cells. At the end of incubation, erythrocytes were collected by centrifugation at 5000 x g for 15 min and washed in isotonic medium. The pellet was used for enzyme determinations. It was suspended in H_2O containing DNase and sonicated at a 50 w power for 1 min to perform hemolysis. Cell debris was removed by centrifugation at 15000 x g for 15 mm, the hemolysate was diluted as previously described (Roche et al. 1991). Peroxidases and catalase activities were determined as previously reported (Roche et al. 1991; Gwozdzinski et al. 1992). Total peroxidase activity was estimated by gaiacol test (25°C pH 7). Catalase activity was measured by the disappearance rate of H_2O_2 monitored at 240 nm (25°C pH 7) and GSH-Px activity, using the consecutive glutathione reductase reaction and oxidation of NADPH, with cumene hydroperoxide as substrate (25°C pH 7.6). SOD activity was measured by its ability to inhibit oxidation of luminol after hypoxathine-xanthine oxidase reaction (25°C, pH 7.8). The amount of MnSOD was analysed in presence of 1 mM cyanide to suppress Cu/Zn SOD activity. Enzyme activities were related to cell haemoglobin content carried out by the cyanomethemoglobin procedure (Sigma diagnostic kit, ref. 525-A). Hemolysis was estimated by the measurement of the haemoglobin in the supernatent ATP content was determined, before and after incubation, in total cell suspension (Boehringer Mannheim kit, ref. 124 885).

Xenobiotics were phenol and monosubstituted phenols (hydroxyphenols, alkylphenols and nitrophenols) (see Figure 1). Phenol (C_6H_4OH); hydroquinone (1,4 dihydroxybenzene), resorcinol (1,3 dihydroxybenzene) and pyrocatechol (1,2 dihydroxybenzene) were purchased from Riedel-de Haën (D-30 16 Seelze 1, Germany). O-cresol (2-CH_3-phenol), o-ethylphenol (2-C_2H_5-phenol), o-propylphenol (2$CH_3CH_2CH_2$-phenol) and ortho-, meta-, para- nitrophenol (2-, 3-, 4-NO_2-phenol) were supplied by Aldrich-Chimie Europe (38070 St Quentin Fallavier, France). Marine salts, other chemical products were purchased from Sigma Chemical Europe (38297 St Quentin Fallavier, France). The partition coefficient (Kow) of a chemical refers to the ratio of its solubility in octanol and in water. Most octanol-water partition coefficients were obtained from the

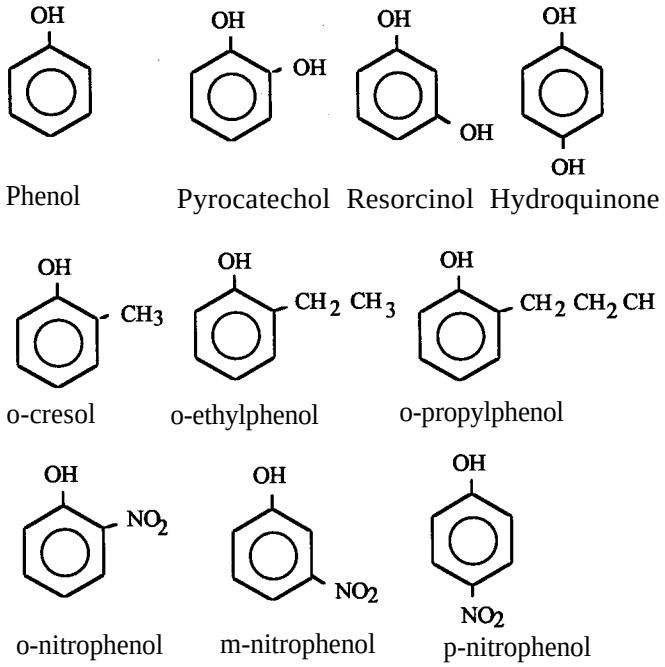

Figure 1. Molecular structure of the phenolic compounds

compilation of Leo et al. (1971) and publications of Schultz and collaborators (see bibliography). The partition coefficients of several substituted phenols were therefore experimentally determined according to the guidelines given by OCDE (1981) and by chemical techniques described by Pesez (1990). The dissociation constant pKa of compounds is used as a second molecular descriptor. The indices of dissociation values, pKa, were taken from Schultz (1987). All determinations reported in the text are the average of five or ten preparations (means ± S.E.M.). For statistical analysis the Statview program was used on a Macintosh computer. P paired tests were used for each chemical. When differences of variances were large, statistical differences were checked using Wilcoxon's two tailed test with a confidence range of $p<0.05$. Correlations were assessed using the Spearman test.

RESULTS AND DISCUSSION

In control cells, hemolysis following 24-hr incubations at 20°C was very low (less than 1%) (Table 1). In phenol treated cells, no significant differences with control were found concerning this parameter. Hydroxyphenols, on the contrary, strongly affected the viability of red blood cells. Hydroquinone and pyrocatechol were the more potent inhibitors since almost 70 % of the red blood cells was hemolysed following 24-hr incubations at 1 mM. At 100 µM, approximately 10% hemolysis remained, which did not completely disappear at 10 µM, especially for pyrocatechol. Resorcinol leads also to a noticeable hemolysis which was however

Table 1. Descriptor data and cytotoxicity on sea bass erythrocytes for phenolic compounds.

Compounds (see Fig. 1)	Physicochemical parameters			Viability test		
	Log Kow bibliography	Log Kow measured	pKa	No of measures	Loss of ATP%	Hemolysis %
Control	-	-		10	23.9±3.6	0.30±0.12
Phenol 1 mM	1.64 ±0.03	1.46	9.92	8	16.8±7.3	0.21±0.07
100 µM	(38)			5	26.1±12.0	0.44±0.09
Hydroqu.1 mM	0.52 ±0.03	0.59	9.55	8	96.6±0.3^s	77.0±2.3^s
100 µM	(11)			5	83.7±0.8^s	8.09±0.11^s
10 µM				5	42.2±7.1^s	0.20±0.10
Resorcin. 1mM	0.73 ±0.03	0.80	9.33	8	42.4±4.3^s	1.18±0.32
100 µM	(8)			5	25.1±9.3	1.06±0.33
Pyrocat. 1 mM	0.86 ±0.09	1.01	9.62	5	96.4±0.1^s	70.0±2.2^s
100 µM	(10)			5	87.4±2.3^s	9.05±0.69^s
10 µM				5	13.9±9.1	2.35±0.66
Cresol 1 mM	2.13 ±0.07	1.95		5	70.6±0.8^s	11.5±5.4^s
100 µM	(10)			5	27.1±9.9	0.03±0.03
Ethylph. 1 mM	-	2.47		5	85.7±2.2^s	37.6±8.3^s
100 µM				5	27.5±11.6	0.88±0.29
Propylph. 1mM	-	3.25		5	99.3±0.1	100.0
100 µM				5	0.7±0.6^s	0.66±0.04
10 µM				5	51.7±6.3^s	0.02±0.01
2-nitroph. 1 mM	2.11 ±0.22 (8)	2.00	6.80	5	34.9±6.7	0.71±0.36
100 µM				5	1.8±7.9^s	0.55±0.05
10 µM				5	36.8±4.7	0
3-nitroph. 1 mM	1.92 ±0.05	1.96	8.25	5	74.4±3.2^s	2.70±0.21
100 µM	(9)			5	45.1±3.6^s	0.66±0.09
10 µM				5	64.5±3.7^s	0
4-nitroph. 1 mM	1.77 ±0.06	1.79	7.15	5	73.4±2.2^s	1.61±0.81
100 µM	(13)			5	81.1±1.7^s	0.19±0.04
10 µM				5	52.7±5.0^s	0.24±0,14

For physicochemical parameters (n) : number of data. Values represent mean±SE.
^{s}: $p<0.05$ vs control

lower than with the other diphenols. The data obtained with ATP corroborated the results of hemolysis with phenol and hydroxyphenols. In control cells, the variation of ATP content following 24-hr incubations, represented approximately 30% the initial value. One mM hydroquinone and 1 mM pyrocatechol were responsible of an almost complete dissipation of cell ATP. At 100 µM, the effect of these compounds was always manifest, since the decrease of ATP content was near 90%. No significant effect of pyrocatechol was found at 10µM whereas hydroquinone still remained potent. Resorcinol also influenced energetic metabolism but

Table 2. Enzymatic activities of sea bass erythrocytes treated with phenolic compounds.

Compounds (see Fig. 1)	Enzymatic activity				
	SODs U.g Hb^{-1}	MnSOD U.g Hb^{-1}	Catalase U.mg Hb^{-1}	Peroxidase U.mg Hb^{-1}	GSH-Px U.g Hb^{-1}
Control	114.7±5.8	13.5±0.8	134.7±10.4	3.76±0.15	18.7±1.4
Phenol 1 mM	135.3±7.9	17.0±0.6	170.1±27.9	4.53±0.31^s	14.3±1.5
100 µM				3.66±0.06	
Hydroqu. 100 µM	97.8±9.5	19.7±1.5	108.0±5.3	3.60±0.06	22.5±3.0
Resorcin. 1 mM	121.9±10.5	16.3±1.8	132.2±19.2	5.35±0.42^s	9.2±1.3^s
100 µM				3.73±0.10	22.4±0.5
Pyrocat. 100 µM	131.3±4.1	24.6±0.7^s	46.6±3.2^s	4.30±0.07^s	29.5±5.4^s
10 µM		19.7±0.1^s	112.4±6.9	3.58±0.12	25.5±0.6
1 µM		13.4±0.7			
Cresol 1 mM	123.7±13.9	17.3±0.8	117.5±9.1	3.89±0.08	30.4±9.4^s
100 µM					30.4±2.8^s
10 µM					19.5±0.2
Ethylph. 100 µM	130.5±7.2	22.3±4.3^s	104.5±9.3	3.6±0.2	15.9±1.0
10 µM		17.4±0.3			
Propylph. 100 µM	101.1±5.5	15.3±1.3	110.2±2.8	3.46±0.05	18.8±2.4
2-nitroph. 1 mM	138.2±11.4^s	17.8±0.2	121.8±3.5	4.10±0.15	18.0±4.5
100 µM	94.0±3.4				
3-nitroph. 1 mM	132.7±9.4	20.7±5.5^s	124.7±14.1	3.94±0.13	3.3±0.2^s
100 µM		16.6±2.5			22.9±0.8
4-nitroph. 1 mM	148.9±12.3^s	21.4±0.2^s	126.8±1.7	3.92±0.06	17.5±6.7
100 µM	115.4±6.0	18.3±1.9			

Enzymes activities are reported as units/g or mg hemoglobin. Values represent means ± SE of 5 to 10 samples.

^{s}: $p<0.05$ vs control

to a lower extent, since a 40 % decrease of ATP content has been shown at 1 mM. No effects were observed at 100 µM. The enzymes involved in the metabolism of oxygen active derivatives were diversely influenced according to the chemical structure of the toxicant (Table 2). The more notable effects of diphenols have been found with pyrocatechol which increased the activities of MnSOD, peroxidase and GSH-Px at 100 µM. This stimulation persisted at a ten times lower concentration for MnSOD activity. Conversely catalase activity decreased. Such an issue suggests that diphenols, and especially pyrocatechol, interacts with the production of oxygen radicals. This hypothesis is supported by the fact that during autoxidation of some diphenols such as hydroquinone, such free radicals are formed (Eyer 1991). Moreover, in higher vertebrates, hydroxyphenols oxidise haemoglobin to methemoglobin with a

concomitant hyperproduction of oxygen active species. These radicals could be responsible of cell membrane damage leading to rapid hemolysis as it has been previously suggested for copper (Roche and Bogé 1993).

At the highest concentration, the hemolysing capacity of alkyl compounds with an ortho side carbon chain was much more notable than that of phenol. This effect is clearly related to the length of this carbon chain. In particular, it can be noticed that 1 mM propylphenol was responsible of a total cell hemolysis. Cell ATP was also dependent on this ortho carbon chain, the lowest content being found in cells treated with 1 mM propylphenol. It can be noticed that low ATP levels were still observed with 10 uM propylphenol. Paradoxically, an intermediate concentration of this chemical (100 µM) seems to protect the cells from ATP depletion. In contrast to diphenols, alkylphenols had only minor effects on enzyme activities. Cresol was responsible for an increase of GSH-Px activity, whereas other compounds had no notable effect.

Nitrophenols had a lower hemolysing activity than the other phenolic substitutes. Nevertheless, these compounds were more cytotoxic than phenol as shown by a severe decrease of cell ATP obtained on cell treated with a very low concentration (10 µM). At 10 µM and 100 µM, a typical structure-activity relationship was found, 4-nitro and 3-nitro being more active than 2-nitrophenol. The more significant effects of nitrophenols on enzyme activities were concerned with GSH-Px: the activity of which was strongly depressed by 1 mM 3-nitrophenol. Moreover, these compounds were responsible for a slight increase of total SOD and Mn SOD activities. These activities were no longer affected using a ten times lower concentration.

Numerous assays have been performed in order to correlate the molecular structure of a chemical with its biological activity expressed notably in term of toxicity. The more common descriptor is the octanol/water partition coefficient (Kow) which is used to predict the accumulation of the chemical in biological tissues (Neeley et al. 1974). The partition coefficient decreases for phenols having a second hydroxyl group (hydroxyphenols). It increases with the length of the aliphatic chain of alkylphenols, whereas it seems not influenced by the position of the NO_2 of nitrophenols. This work demonstrated that the viability responses (hemolysis, cell ATP levels) are good tools for monitoring the effects of phenolic compounds on fish erythrocytes. A correlation between the logarithm of this partition coefficient and the logarithm of these parameters has been attempted. When the phenolic compounds are considered together, no significant relationship can be established. However, such a relationship exists within some phenol families, notably alkylphenols. The cytotoxicity of these chemicals increases with the number of carbon atoms and with the partition coefficient.

Log (% hemolysis) = - 0.3 1 + 0.72 Log Kow $r^2 = 0.991$, n=3
Log (% loss ATP) = - 1.65 + 0.11 Log Kow $r^2=0.927$, n=3.

Phenol and alkyl substituted derivatives are polar narcotics (Lipnick et al. 1988; Xu et al. 1994) and for such compounds a simple model for toxicity was developed based on computed estimates of the physicochemical properties notably on log Kow (Baker et al. 1988 quoted from Xu et al. 1994). Although the sample size is

small, our results suggest that for such compounds the partitioning coefficient can be used to predict the cell toxicity on fish erythrocytes. For other compounds, another descriptor has been assayed such as pKa. Indeed, toxic effects are generally enhanced when the chemical is in a ionised form (Schultz 1987). Unfortunately, as for the partition coefficient, no relationship exists for fish erythrocytes, between the pKa (or pKa + log Kow) of all substituted derivatives of phenol and their cytotoxicity. Such a correlation could be found within hydroxyphenols and nitrophenols. Indeed, among hydroxyphenols, resorcinol has the lowest pKa and the lowest cytotoxicity and among nitrophenol, 3- and 4-nitrophenol have higher pKa and higher cytotoxicity than 2-nitrophenol. However, due to the low range of the pKa within each group, this interpretation requires great caution. The difficulty to draw a simple relationship between some physicochemical properties of phenolic compounds and their effects on fish erythrocytes can be explained by the structural features of some of them which are likely to cause underlying behavior. This is typically the case of pyrocatechol and hydroquinone which tautomerize and form the reactive quinone moiety with possible specific effect on membrane, as previously thought. But this is also the case of nitrophenols, notably the ortho and para derivatives which tautomerize and form Michael-type acceptors (Roberts 1987).

This work has shown that the toxicity of phenols is strongly dependent upon their structure (Jaworska and Schultz 1991). Diphenols, nitrophenols and alkylphenols lead to a higher cytotoxicity than phenol. These compounds are strong inhibitors of the energetic metabolism (Cajina-Quezada and Schultz 1990). With regard to this specific criteria, the more active compounds are nitrophenols, such as 4-nitro, and hydroxyphenols, notably pyrocatechol. Moreover phenols lead also to severe hemolysis, pyrocatechol, hydroquinone and propylphenol being the more powerful agents. The question arises concerning the origin of this membrane disruption which can result either from the depletion of ATP or from specific effects on membrane structures (Keweloh et al 1991). In the case of alkylphenols, these damages could be related to narcotic properties and interaction with lipid bilayers, whereas for diphenols they could be due to an imbalance of the metabolism of active oxygen species (Canada and Calabrese 1989). In connection with that, pyrocatechol seems to be the more effective agent to induce an increase in enzyme activities involved in cell protection towards oxygen active derivatives, even at low concentrations (10 µM).

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