Lipophilicity profiling and cell viability assessment of a selected panel of endocrine disruptors

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Abstract

Endocrine disruptors are chemicals widely used worldwide by industries in a variety of applications. Routinely exposure to these chemicals, even if at low doses, can cause damage effects on human health. In the present study we evaluated toxic effects of nine chemicals, among which phthalates, using various cell lines to investigate their capability to interfere with cell proliferation and viability.

Alongside, we investigated their affinity for phospholipids to assess the possible passage through biomembranes. Experimentally determined $\log k_w^{IAM.MG}$ values ranged from 1.37 to 3.49 whilst calculated $\log k_w^{IAM.DD2}$ spanned from 1.80 to 5.21, supporting the target contaminants to exhibit lipophilicity to moderate to very high. The achieved results were related to pharmacokinetic and

toxicological properties by ADMET predictor™ and EPI Suite™ software. Triclosan and 4-Nonylphenol were found to be the most toxic against all cell lines screened, showing an IC_{50} of 30 μM for triclosan on human keratinocytes and of 50 μM for 4-Nonylphenol on human colorectal adenocarcinoma cells. Overall, even if the phthalates showed higher IC_{50} values (ranging from 170 μM to 280 μM), we can assert that all contaminants herein tested were able to interfere with cell growth and viability.

1. Introduction

Contaminants of emerging concern (CECs) are a class of chemicals that are gathering the attention of the scientific community due to their ubiquity and persistence in the environment, and their remarkable bio-accumulative and toxic potential. CECs can be included intentionally or unintentionally in various consumer goods routinely handled by people and identify a broad and diversified class of compounds including drugs, chemicals contained in personal care products, agents used for household cleaning and agricultural purposes, additives exploited in industrial processes. Among CECs, phthalates (PEs), esters of phthalic acid, are chemicals used to aid the dissolution of other materials (Annamalai and Vasudevan, 2020), since their presence makes plastics more durable, flexible, and transparent. Bis(2-ethylhexyl) phthalate (DEHP) and Diisononyl phthalate (DINP) are currently the most widespread PEs, although, over the years, about a dozen of new phthalates have been synthesized, including Diisodecyl phthalate (DIDP) and Dibutyl phthalate (DBP) (Wang et al., 2019; Dutta et al., 2020). High molecular weight (MW) PEs, such as DEHP and DINP are employed in the manufacture of hundreds of plastic products, such as building materials, adhesives, and flexible vinyl including flooring and wall coverings.

Low MW PEs, such DBP, are used in personal care products such as cosmetics, shampoos, skin moisturizers, hair sprays, nail polish and even in food packaging, instead. Regardless their MW, PEs

pose a potential health concern because these can exert a disruptive effect on the endocrine system of living organisms, due to their action in affecting the hormone homeostasis (Zarean et al., 2016; De Toni et al., 2017; Zhu et al., 2018; Zhang et al., 2021). Lower MW PEs are rapidly absorbed through the diet and by inhalation (Başaran et al., 2020; Li et al., 2021), instead PEs with longer alkyl side chains exhibit low dermal absorption, according to several toxicokinetic studies (Fromme, 2011; Giuliani et al., 2020). Many scientific reports support that PEs can exert adverse effects on liver, kidneys, and the reproductive system (Kim et al., 2018; Karwacka et al., 2019; Wang and Qian, 2021).

The aim of our research was the investigation of toxicity of five PEs widely used by industries along with other endocrine disrupting chemicals (EDCs), such as Triclosan, (TCS), 1,2,4,5-Tetrachlorobenzene (TCB), 1,4-Dichlorobenzene (DCB), 4-Nonylphenol (4-NP), belonging to other chemical classes with lipophilicity similar or lower than that of the phthalate group. The chemical structures of the target compounds are showed in Figure 1. To gain access to the receptor site, and, therefore, trigger harmful effects in the human body, EDCs must necessarily cross the biological barriers. For this reason, we explored the affinity for membrane phospholipids of all nine chemicals by Immobilized Artificial Membrane (IAM) Liquid Chromatography (LC) technique (Barbato et al., 2004)(Grumetto and Russo, 2021), yielding log k_w^{IAM} values, *i.e.* a phospholipophilicity measure. This method allows to depict distinct interactions, mainly of electrostatic nature, that are different from those encoded by the "classical" lipophilicity. It represents a crucial parameter for describing passive transcellular diffusion through biomembranes, that is traditionally expressed by the logarithm of the ratio of analyte concentrations in an organic solvent, usually *n*-octanol, and an aqueous phase (log P) (Liu et al., 2011). Moreover, we used the software ADMET Descriptor™ (Ghosh et al., 2016) and Estimation Program Interface (EPI Suite™), to explore possible relationships between phospholipophilicity and pharmacokinetics parameters as well as ecotoxicological endpoints of all nine considered molecules. Indeed, recently scientific evidence supported that exposure to CECs such

as EDCs could contribute to the pathogenesis of metabolic diseases. In particular, they could cause developmental and reproductive toxicity, hepatotoxicity, neurotoxicity, and even carcinogenesis (Pan et al., 2006; Meng et al., 2014). In order to evaluate cellular toxicity potential of EDCs under investigation in this research work, we carried out *in vitro* experiments in terms of interference with cell growth and proliferation on a restricted panel of well-established human cells, including both healthy and cancer cell lines.

2.Materials and Methods

2.1 Chemicals

Acetonitrile (minimum purity ≥95.0%) was purchased from Sigma Aldrich (Milan, Italy). Bis(2-ethylhexyl) phthalate (DEHP- minimum purity ≥ 98.0 %), Diisononyl phthalate (DINP- minimum purity ≥ 99.0 %), Diisodecyl phthalate (DIDP- minimum purity ≥ 99.0 %), Dibutyl phthalate (DBP-minimum purity ≥ 99.0 %), Dioctyl phthalate (DnOP- minimum purity ≥ 98.0 %), 4-nonylphenol (4-NP-minimum purity ≥ 98.0%), 1,4-dichlorobenzene (DCB-minimum purity ≥ 99.0 %), 1,2,4,5-tetrachlorobenzene (TCB-minimum purity ≥ 98.0%), Triclosan (TCS-minimum purity ≥ 97.0%) were purchased from Sigma-Aldrich (Dorset, United Kingdom).

2.2 IAM chromatography

For DCB, TCB and TCS, phospholipophilicity, measured as log k_w^{IAM}, was experimentally determined by high-performance liquid chromatography (LC-20 AD VP; Shimadzu Corp., Kyoto, Japan), equipped with an ultraviolet (UV)–visible detector (Shimadzu Model SPD10 AV) set at λ 220 nm, analytical column IAM.PC.MG (4.6 × 150 mm; Regis Chemical Company, Morton Grove, IL). Mobile phases were vacuum filtered through 0.45 μm nylon membranes (Millipore, Burlington, MA USA). Data acquisition and integration were accomplished by Cromatoplus software for personal

computer. Employed eluents were 0.1 M phosphate buffer at pH 7.0 and acetonitrile at various percentages with a flow rate ranging from 1.0 to 2.3 mL min^{-1}. All samples were dissolved in acetonitrile (ca. 10^{-4} M) and chromatographic analysis were carried out at 22 ± 2° C. Affinity of the chemicals for the IAM.PC.MG was measured as retention factor extrapolated at 100 % of aqueous phase (k_w^{IAM}) by performing a polycratic method of extrapolation (Braumann et al., 1983). Since all the compounds under our investigation required at least the addition of acetonitrile to mobile phase to elute within 20 min, three different mobile phases containing acetonitrile in percentages (φ) ranging from 10% to 30% (v/v) were employed. All values of log k_w^{IAM} are the average of at least three measurements.

2.3 IAM calculation

Six of the studied analytes were not experimentally accessible on the IAM.MG stationary phase, while all compounds were again not experimentally accessible on the IAM.DD2 phase. For this reason, the remaining analytical values were predicted *in silico* via the correlative equations reported in (Russo et al., 2017). The tool (available at https://www.ddl.unimi.it/vegaol/logkwiam.htm) offers a calculation of k_w^{IAM} values on both IAM.PC.MG and IAM.PC.DD2 columns, of any molecule included in the PubChem collection as implemented in the script version. The software is based on 205 log $kw^{IAM.MG}$ and 161 log $kw^{IAM.DD2}$ values experimentally achieved by LC.

2.4 In silico prediction of ADMET properties and ecotoxicological endpoints

As the nine chemicals under our investigation differ in physicochemical, as well as absorption, distribution, metabolism, excretion, and toxicity (ADMET) profile, we used ADMET Predictor™ software for Windows-based personal computers, (version 8.1.0.11, Simulations Plus, Lancaster, CA, USA), as tool applicable for the estimation of ADMET properties. ECOSAR module of EPI Suite™ Software (Version 4.11., 2012. U.S. Environmental Protection Agency, Office of Pollution, Prevention and Toxics: Washington) was employed to calculate ecotoxicological endpoints as pLC50

values (96-h) for fish. EPI environmental software is a predictive tool, freely available at E.P.A. (U.S. EPA, 2012), estimating aquatic toxicity-acute (short-term) and chronic (long-term) of chemicals in aquatic organisms, among which fishes.

2.5 Cell cultures

Human breast adenocarcinoma cells MCF-7 (Endocrine-Responsive, ER), human ovarian cancer cells A2780 and human keratinocytes HaCaT were grown in DMEM (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), L-glutamine (2 mM, Sigma, Milan, Italy), penicillin (100 units/ml, Sigma) and streptomycin (100 µg/ml, Sigma), and cultured in a humidified 5% carbon dioxide atmosphere at 37 °C. Human colon adenocarcinoma cells Caco-2 were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 20% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), and cultured in a humidified 5% carbon dioxide atmosphere at 37 °C. Moreover, Caco-2 cells, led to differentiation, were used at post-confluence stage as a model of human enterocytes (Di Cesare Mannelli et al., 2018). All cell lines were grown according to ATCC recommendations.

2.6 Treatments and cell toxicity assessment

The cytotoxic activity of selected contaminants was investigated through the estimation of a "cell survival index", arising from the combination of cell viability evaluation with cell counting (Russo et al., 2021). Cells were inoculated in 96-microwell culture plates at a density of 10^4 cells/well and allowed to grow for 24 h. The medium was replaced with fresh medium and cells were treated for further 48 h with a range of concentrations (10 → 200 µM) of each chemical under our study. Cell viability was evaluated using the MTT assay, which measures the level of mitochondrial dehydrogenase activity using the yellow 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) as substrate. The assay is based on the redox ability of living mitochondria to convert dissolved MTT into insoluble purple formazan. Briefly, after the treatments, the medium

was removed, and the cells were incubated with 20 µL/well of a MTT solution (5 mg/mL) for 1 h in a humidified 5 % CO_2 incubator at 37 °C. The incubation was stopped by removing the MTT solution and by adding 100 µL/well of DMSO to solubilize the obtained formazan. Finally, the absorbance was monitored at 550 nm using a microplate reader (iMark microplate reader, Bio-Rad, Milan, Italy). Cell number was determined by TC20 automated cell counter (Bio-Rad, Milan, Italy), providing an accurate and reproducible total count of cells and a live/dead ratio in one step by trypan blue exclusion assay. Bio-Rad's TC20 automated cell counter uses disposable slides, TC20 trypan blue dye (0.4 % trypan blue dye w/v in 0.81 % sodium chloride and 0.06 % potassium phosphate dibasic solution) and a CCD camera to count cells based on the analyses of captured images. Once the loaded slide is inserted into the slide port, the TC20 automatically focuses on the cells, detects the presence of the trypan blue dye, and provides the count. When cells are damaged or dead, trypan blue can enter the cell allowing living cells to be counted. After chemical exposure in 96-microwell culture plates, the medium was removed, and the cells were collected. 10 µL of cell suspension, mixed with 0.4 % trypan blue solution at 1:1 ratio, were loaded into the chambers of disposable slides. The results are expressed in terms of total cell count (number of cells per mL). If trypan blue is detected, the instrument also accounts for the dilution and shows live cell count and percent viability. Total counts and live/dead ratio from random samples for each cell line were subjected to comparisons with manual hemocytometers in control experiments. The calculation of the concentration required to inhibit the net increase in the cell number and viability by 50% (IC50) is based on plots of data (n = 6 for each experiment) and repeated five times (total n = 30). IC50 values were obtained by means of a concentration response curve by nonlinear regression using a curve fitting program, GraphPad Prism 8.0, and are expressed as mean values ± SEM (n = 30) of five independent experiments.

2.7 Statistical analysis

All data were presented as mean values ± SEM. The statistical analysis was performed using Graph-Pad Prism (version 8.0, Graph-Pad software Inc., San Diego, CA) and ANOVA test for multiple comparisons was performed followed by Bonferroni's test.

3. Results and Discussion

Keeping in mind that several environmental contaminants have been associated with biological processes leading to adverse human health consequences, the nine chemicals *i.e.*, 5 phthalates, 2 chlorobenzenes, 4-Nonylphenol and Triclosan were tested for their ability to interfere with cell viability. In particular, their *in vitro* biological effects on a selected panel of healthy and tumor cell lines (HaCaT, enterocytes, MCF-7, A2780 and Caco-2) were investigated, as reported in Figures 2 and 3 and Table 1, the latter showing IC_{50} values after 48 h of incubation *in vitro*.

Cell toxicity data of the panel of contaminants under investigation were consistent in indicating that TCS and 4-NP are the most toxic compounds inducing strong effects in all the *in vitro* models used, showing IC_{50} values in the low micromolar range. TCS has shown toxic effects on HaCaT keratinocytes (IC_{50} of 30 μM), on MCF-7 breast cancer cells (IC_{50} of about 45 μM) and on Caco-2 cells (IC_{50} of about 50 μM), whereas on enterocytes and Caco-2 cells IC_{50} values were at 50 μM. 4-NP has shown toxic effects starting from 50 μM on all cell lines tested. Previous studies demonstrated that TCS-induced toxicity is related to its capacity to dysregulate the redox status of cells, leading to oxidative damage of lipids, proteins, and DNA (Binelli et al., 2009; Park et al., 2016; Zhang et al., 2018). Moderate toxicity was found for TCB on MCF-7 breast cancer cells and for DCB on HaCaT cells. Among the phthalates, the toxic effects were moderate. DBP and DEHP moderately interfered with MCF-7 cells viability. Moreover, we have also observed a contaminants-induced proliferative effect. In particular, DNOP and DIDP induced a significant increase in A2780 cells proliferation after 48h of treatment at 50 μM, whereas a significant increase of proliferation of enterocytes was observed after 48h of DEHP exposure at 50 μM. Overall, our results agree with the

191 literature (Lepretti et al., 2015; Rubin and Zucker, 2022) that assessed TCS and 4-NP cause a decrease
192 in cell viability of Caco-2 cells. On MCF-7 cells our results for 4-NP treatment agree with Vivacqua
193 et al. (Vivacqua et al., 2003), that demonstrated an important inhibition of cell proliferation at 100
194 μM, although at low concentrations 4-NP induced cell proliferation. Similarly, Yoon et al. (Yoon and
195 Kwack, 2021) reported a proliferative effect of TCS on MCF-7 cells, suggesting that the
196 contaminants-induced proliferative effects could be associated with estrogenic activity interference.

197 The results regarding phospholipophilicity values of the nine chemicals under analysis, were
198 expressed as log $k_w^{IAM.MG}$ and log $k_w^{IAM.DD2}$ values either calculated *in silico* or experimentally
199 measured on IAM.PC.MG stationary phase. Table 2 shows all data, as well as log P values.

200 IAM.LC approach, both as experimental approach and *in silico* predicted values, was chosen as the
201 log P determination by the traditional "shake-flask" method was poorly reproducible, due to the very
202 high lipophilicity values, up to 10.36 of DIDP. Actually, IAM.LC can be considered as a technique
203 for the determination of the affinity xenobiotics/biomembranes and therefore of their potential of
204 crossing of biological barriers. Indeed, the IAM stationary phases consist of analogues of
205 phosphatidylcholine (PC), one of the main constituents of biological membranes. Unfortunately,
206 despite we tried shorter column formats (as short as 1 cm), it has not been possible to carry out the
207 determination also on IAM.PC.DD2 stationary phase, due to the very high retention time of the
208 analytes, which were unsuitable as (phospho) lipophilicity indexes (Taillardat-Bertschinger et al.,
209 2003).

210 IAM.PC.DD2 differs from IAM.PC.MG in the end-capping of residual amino groups of the silica-
211 propylamine core supporting C_{10} and C_3 alkyl chains being end-capped by both decanoic and
212 propionic anhydrides, while IAM.PC.MG supports hydroxy groups, being end-capped by methyl
213 glycolate. The different chemistry of the end-capping makes the stationary phase IAM.MG more
214 polar than IAM.DD2, and better suited for the profiling of these contaminants. Anyway, in all the

other works by our group, IAM parameters measured on both stationary phases, were found to be strongly interrelated (Grumetto et al., 2012; Grumetto et al., 2016; Russo et al., 2017). This is accurate even so more with the compounds belonging to this panel, as all of them are essentially uncharged at the experimental conditions (pH 7.0). Indeed, log kw^{IAM} values for structurally unrelated neutral compounds, was found to unambiguously relate with the respective *n*-octanol/water partition coefficients (log P), as no electrostatic interactions, which are distinctive of the IAM phase as compared to *n*-octanol/water, can occur between the analytes and the electrically charged stationary phase (Grumetto et al., 2016). The log k_w^{IAM} values of DCB, TCB and TCS was achieved experimentally on IAM.PC.MG, while the log k_w^{IAM} values of the remaining chemicals were calculated, as inaccessible even at maximum percentage of organic solvent, *i.e*. 30% (v/v) allowed. IAM indexes (log k_w^{DD2}) were related to all ADMET Predictor™ parameters. This software includes several built models based on a statistical methodology that generates, starting from molecular structure, physicochemical properties with a high degree of accuracy. Designed using artificial neural network ensemble (ANNE) models (Paixao et al., 2014), it generates 140 *in silico* calculated properties and predicts toxicological endpoints. Among ADMET™ output, we selected those with the best scores; four parameters were found quite well related, suggesting a link between phospolipophilicity and the modulation of the passage through cell membranes, that is a prerequisite for causing toxicity: a) ADMET Risk, indicating the number of potential ADMET problems a compound might have (score range 0-22), b) Absorption Risk, indicating the number of potential oral absorption problems a compound could have (score range 0-8), c) LD50 for rat acute toxicity (mg/Kg in oral dose that would be lethal to 50% of the rats), d) DiffCoeff, an Hayduk-Laudie infinite dilution diffusion coefficient (cm^2/s* 105) of nonelectrolytes in water. The best relationships are shown in Figure 4, while the full matrix is reported in supplementary material section (Table S1). PEs show lowest diffusion values, except for DBP, which behaves as an outlier, probably due to its lower phospholipophilicity values and its shorter alkyl chains. IAM.LC measurements relate quite well

with permeability and toxicity data calculated *in silico* and offer information that was complementary to that afforded by cell viability assays.

ECOSAR™, included in EPI Suite™, estimates the acute (short-term) and chronic (long-term or delayed) toxicity of industrial chemicals on aquatic organisms, such as fishes, aquatic invertebrates, and green algae. Predicted pLC_{50} values reported in Table 3 show a good relationship with log kw^{IAM} (r=0.9729), indicating that a higher phospholipophilicity is related to a higher value of toxicity for fishes (Figure 5)**.**

4. Conclusions

In vitro data support a stronger cytotoxic action of two compounds *i.e*., 4-NP and TCS, on all investigated cell lines, both normal and cancerous. This is consistent with similar studies by independent scholar and could be attributed to a perturbation of the redox status of cells, suggesting a universal rather than a tissue specific mechanism of action. The reduced cell viability induced by 4-NP and TCS can be attributed to optimal phospholipophilicity and diffusion values on the contrary of PEs that did not show a significant cytotoxicity. The phospholipophilicity measurements/calculations allowed to achieve a consistent scale of affinity of these compounds for the cellular membranes. These were found to be related to data of ADMET risk and aquatic toxicity, as predicted by specialized software.

Declaration of interest

The authors do not declare any competing financial interest.

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