Science of the Total Environment 917 (2024) 170461

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Into the toxicity potential of an array of parabens by biomimetic liquid chromatography, cell viability assessments and *in silico* predictions

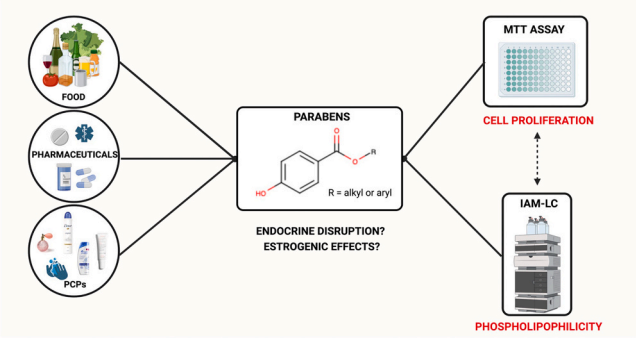
Ilaria Neri [a,b,c], Janis MacCallum [a], Ritamaria Di Lorenzo [b], Giacomo Russo [a,*], Frédéric Lynen [d], Lucia Grumetto [b]

[a] Centre of Biomedicine and Global Health, School of Applied Sciences, Sighthill Campus, Edinburgh Napier University, 9 Sighthill Ct, EH11 4BN Edinburgh, United Kingdom
[b] Department of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, Via D. Montesano, 49, I-80131 Naples, Italy
[c] Consorzio Interuniversitario INBB, Viale Medaglie d'Oro, 305, I-00136 Rome, Italy
[d] Separation Science Group, Department of Organic and Macromolecular Chemistry, Ghent University, Krijgslaan 281 S4bis, B-9000 Ghent, Belgium

HIGHLIGHTS

- Five parabens and their parent compound were characterised.
- Characterisation was performed *in vitro* (cells and chromatography) and *in silico*.
- Chromatography was performed on IAM and protein phases.
- Isopropyl paraben induced the strongest proliferative action on MCF-7 cells.
- Relationship between proliferative effect at 0.01 μM concentration and IAM data.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Lidia Minguez Alarcon

Keywords:
Parabens
Endocrine disrupting chemicals
MCF-7 cell line
ADMET prediction
Immobilized artificial membranes
Biomimetic chromatography

ABSTRACT

Five parabens (PBs) *i.e.*, Methylparaben (MP), Ethylparaben (EP), Isopropylparaben (iPrP), Isobutylparaben (iBuP), Benzylparaben (BzP), and their parent compound *i.e.*, para–hydroxy Benzoic Acid (pHBA), were studied both *in vitro* and *in silico*. Specifically, we determined their retention on several both protein- (Human Serum Albumin and α_1-acidic glycoprotein) and (phospho) lipid- (immobilized artificial membrane (IAM)) based biomimetic stationary phases to evaluate their penetration potential through the biomembranes and their possible distribution in the body. The IAM phases were based either on phosphatidylcholine (PC) analogues *i.e.*, PC.MG and PC.DD2 or on sphingomyelin (SPH). We also assessed their viability effect on breast cancer cells (MCF-7) *via* MTT assay subjecting the cells to five different PB concentrations *i.e.*, 100 μM, 10 μM, 1 μM, 0.1 μM and 0.01 μM. Finally, their pharmacokinetics and toxicity were assessed by the ADMET Predictor™ software.

Isopropylparaben was found to be more active than 17β estradiol (E2) employed as positive control, on the screened cell line inducing cell proliferation up to 150 % more of untreated cells. Other analogues showed only a slight/moderate cell proliferation activity, with parabens having longer/branched side chain showing, on average, a higher proliferation rate. Significant linear direct relationships (for PC.DD2 $r^2 = 0.89$, $q^2 = 0.86$, for

* Corresponding author.
E-mail address: G.Russo@napier.ac.uk (G. Russo).

https://doi.org/10.1016/j.scitotenv.2024.170461
Received 6 December 2023; Received in revised form 24 January 2024; Accepted 24 January 2024
Available online 28 January 2024

SPH $r^2 = 0.89$, $q^2 = 0.85$, for both P value < 0.05) were observed between the difference in proliferative effect between the readout and the control at 0.01 μM concentration and the retention on the IAM phases measured at pH 5.0 for all compounds but pHBA, which is the only analyte of the dataset supporting a carboxylic acid moiety. IAM affinity data measured at pH 7.0 were found to be related to the effective human jejunal permeability as predicted by the software ADMET® Predictor, which is relevant when PBs are added to pharmaceutical and food commodities.

Abbreviation list

CECs	contaminants of emerging concern
PCPs	Personal care products
E2	17β estradiol
EDCs	Endocrine disrupting chemicals
PB	Paraben
pHBA	p-hydroxybenzoic acid
IAM.LC	Immobilized artificial membrane liquid chromatography
HSA	Human Serum albumin
AGP	Alpha-1 acid glycoprotein
MP	Methyl 4-hydroxybenzoate
EP	Ethyl 4-hydroxybenzoate
iPrP	Isopropyl 4-hydroxybenzoate
BzP	Benzyl 4-hydroxybenzoate
iBuP	Isobutyl 4-hydroxybenzoate
DMSO	Dimethylsolfoxide
dH_2O	distilled water
FBS	foetal bovine serum
PBS	phosphate buffer saline
P_{eff}	Effective human jejunal permeability (cm/s × 10ˆ4). RMSE/MAE = 0.31/0.25 log units
PPs	plasma proteins
SPH	Sphingomyelin
diff coeff	Hayduk-Laudie infinite dilution diffusion coefficient ($cm^2/s \times 10^5$) of nonelectrolytes in water
LogBB	Logarithm of the Brain/Blood partition coefficient. RMSE/MAE = 0.37/0.28 (2D and 3D)
MTT assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay
MCF-7	breast cancer cell line (Michigan Cancer Foundation-7)

1. Introduction

For the vast majority, pollutants have an anthropogenic source and are ubiquitous in the environment. Amongst these, in the last decades, contaminants of emerging concern (CECs) (Yadav et al., 2021) have attracted the attention of the scientific community due to pollution control, and in an attempt to implement strategies to limit their release in the environment. Humans are widely exposed to these chemicals as they occur in water, air, and soil. Exposure occurs mainly through the diet but also through skin contact and inhalation (Niede and Benbi, 2022; Pena-Pereira et al., 2021). In fact, these contaminants can bio accumulate and exert toxicity in animals and humans, even at very low levels of exposure. Indeed, these molecules, the so-called Endocrine Disrupting Chemicals (EDCs), act by mimicking the natural hormones (Carson, 2002). EDCs can be present, intentionally, or unintentionally in products routinely handled or consumed, such as pharmaceuticals, personal care products (PCPs), and housecleaning products.

Indeed, EDCs belong to various chemical classes and therefore show different mechanisms of action. Parabens (PBs) are a group of aliphatic esters of p-hydroxybenzoic acid (pHBA), which are broadly used for the preservation of consumer products due to their chemical stability, low cost, and low risk of inducing allergic reactions (Petric et al., 2021; Torfs and Brackman, 2021). Recent studies demonstrated that PBs act as EDCs and are also considered as CECs, with an adverse impact on the human health (Nowak et al., 2018; Wei et al., 2021). Although many *in vitro* and *in vivo* studies support an acute toxicity of PBs in animals (Lee et al., 2018), little data is available concerning human health hazards. For these contaminants, the exposure-safety profile is hard to establish to an extent that, although these have been demonstrated to be non-carcinogenic, non-teratogenic and non-mutagenic (Petric et al., 2021) for some PBs *e.g.* Methyl 4-hydroxybenzoate (MP) and Ethyl 4-hydroxybenzoate (EP), regulatory bodies have set thresholds that should not be exceeded. Despite these pressing concerns, no suitable replacements of PBs have been identified yet and since the exposure to these chemicals occurs from various routes, additive, synergistic effects (Petric et al., 2021) are likely. After absorption, PBs are metabolically conjugated to glucuronic acid, sulphate, or glycine by liver microsomes, and metabolic rates are inversely proportional to chain length (Wei et al., 2021). PBs are excreted mainly (>90 %) as conjugated, and to a much lower extent as unconjugated (Janjua et al., 2008; Moos et al., 2016). They can be degraded through a base-catalysed hydrolysis of the ester bond to pHBA. This reaction can occur both in the environment after discharge and *in vivo*. When PBs are included as excipients in topical formulations and these are applied on the intact skin, they are rapidly metabolized to the inactive metabolite pHBA or p-hydroxy hippuric acid (Abbas et al., 2010; Shin et al., 2019) by the carboxylesterases in keratinocytes.

To gain insights on their effects on the human health, we investigated the toxicity of five PBs and their parent compound, pHBA (Fig. 1), *in vitro*, in terms of interference with cell viability on human breast cancer cell line, *i.e.* MCF-7, because of the estrogenic-dependent mechanism of breast cancer and the topical exposure of PBs-containing cosmetics in the closely located under arm area (Harvey and Everett, 2012). Indeed, recent studies suggest that PBs can modulate estrogen-converting enzymes, such as 17β-hydroxysteroid dehydrogenase 1 and 2, thus increasing estrogenic levels and pro-oncogenic c-Myc expression in ER+/HER2+ breast cancer cells (Hager et al., 2022). The choice of PB concentrations was determined as toxicologically relevant ranges according to the literature (Byford et al., 2002; Dagher et al., 2012; Gomes et al., 2018; Watanabe et al., 2013) and this was compared to a physiologically relevant reference level of E2 that would have observable effects on the cells. We also investigated if the *in vitro* effect on cell response was influenced by their affinity for biomembranes and plasma proteins. To assess the membrane affinity of PBs, we used Immobilized Artificial Membrane Liquid Chromatography (LC) and explored possible relationships between phospholipids (phosphatidylcholine (PC) and sphingomyelin (SPH)) affinity and MCF-7 cell viability. Finally, because contaminant toxicity is affected also by pharmacokinetic parameters, such as protein binding, we investigated PBs binding to the main plasma proteins (PPs) *i.e.*, Human Serum Albumin (HSA) and Alpha-1 acid glycoprotein (AGP), which represent the most abundant carrier proteins of endogenous and xenobiotic compounds in the bloodstream, employing biomimetic stationary phases featuring these two proteins immobilized onto a silica core. Indeed, especially for hydrophobic compounds, protein binding may play an important role, as strong binding to PPs

typically induces a depot effect, leading to a delayed/sustained release from the body fat (Fanali et al., 2012) and to consequences in the onset of action and on crucial pharmacokinetic (PK) parameters such as elimination half-life and volume of distribution. To the best of our knowledge, this is the first time that cell viability data of PBs are compared to biomimetic LC and *in silico* data in a single, structured study. Both biomimetic LC experiments and *in silico* prediction are faster and more reproducible than cell viability assays. Therefore, this could pave the way of using these techniques to surrogate toxicity values which is particularly relevant for ample datasets of pollutants, if significant relationships between cell viability and either biomimetic LC or *in silico* prediction are observed.

2. Materials and methods

2.1. Chemicals

Methyl 4-hydroxybenzoate (MP), Ethyl 4-hydroxybenzoate (EP) were purchased from Merck & Co. (Poole, United Kingdom). Isopropyl 4-hydroxybenzoate (iPrP) and Isobutyl 4-hydroxybenzoate (iBuP) were purchased from Fluorochem (UK), Benzyl 4-hydroxybenzoate (BzP) from Sigma-Aldrich (Milan, Italy), pHBA was from J&K (USA). The purity of all PBs was equal to or higher than 98 %. Potassium dihydrogen phosphate (purity > 98 %) was sourced from Merck (Darmstadt, Germany). Milli Q water was produced in-house, and its conductivity was 0.055 μS cm^{-1} at 25 °C (resistivity equals 18.2 MΩ·cm). The pH was determined using a pH meter. The buffer was kept at 4 °C until use for a maximum of one week. HPLC grade Acetonitrile (ACN) and Methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human breast cancer cell line MCF-7 cells were from the American Type Culture Collection (Manassas, Virginia, ATCC number: HTB-22, ECACC number: 86012803). Stock solutions of all PBs were made at a 10 mg/mL concentration in analytical grade DMSO and working solutions of 2.0 mL were made from these by diluting them in dH_2O to 1 mM. The working solution was then filter sterilized using a 0.2 μm filter (Merck KGaA, Darmstadt, Germany), and five different concentrations *i.e.*, 400 μM, 40 μM, 4 μM, 0.4 μM, and 0.04 μM prepared in treatment medium to be used in-well at final concentrations of 100.0, 10.0, 1.0, 0.1 and 0.01 μM. Furthermore, a stock solution of 17β-estradiol (E2, purity ≥98 % from Merck & Co (Poole, United Kingdom)) was made at 37 mM in DMSO and diluted down to 1 mM in dH_2O. 17β-estradiol solution (280.0 nM) was prepared in treatment medium from 1 mM 17β-estradiol solution to be used in the MTT assay at an in-well concentration of 70.0 nM. DMSO working solution was prepared at 400.0 μM in the treatment medium. The IAM.SPH column (150 mm × 2.1 mm, 10 mm), was prepared *in house* (Russo et al., 2021; Verzele et al., 2012).

2.2. Chromatographic analysis

For the study of PBs protein binding the stainless-steel columns were a 100 × 4 mm i.d. CHIRALPAK® HSA and a 100 × 4 mm i.d. CHIRALPAK® AGP (both from Chiral Technologies Inc., West Chester, PA, USA). HSA and AGP chromatography were both performed using eluent at various percentages ranging from 95/5 to 80/20 (*v*/v) 20 mM phosphate buffer pH 7.0/2-propanol at flow rate of 0.9 mL min^{-1}. For the study of PBs affinity for phospholipids, an analytical column IAM.PC.MG (4.6 × 150 mm; Regis Chemical Company, Morton Grove, IL) was used. Liquid chromatograph model consisted in both studies of a Shimadzu system (LC-20 VP - Shimadzu Corp., Kyoto, Japan), a 7725 Rheodyne injection valve fitted with a 20 μL loop, an ultraviolet (UV)/visible detector (Shimadzu Model SPD10 AV) set at λ 254 nm wavelength. Data acquisition and integration were accomplished by Cromatoplus® software. For the determinations on the IAM.PC.DD2 (150 mm × 4.6 μm i.d., 10 mm p.s., 300 Å) and IAM.SPH columns, an Alliance HPLC system (Waters Corporation, U.S) with 2690/2695 separation module, 2996 PDA detector, 717 plus autosampler and 2487 dual λ absorbance detector was used at 254 nm wavelength. The methods were set, and chromatograms were recorded by Waters Empower® 3 software. On all the IAM columns, employed eluents were 0.1 M phosphate buffer at pH 7.0 and acetonitrile at percentages between 70/30 (*v*/v) and 95/5 (v/v)

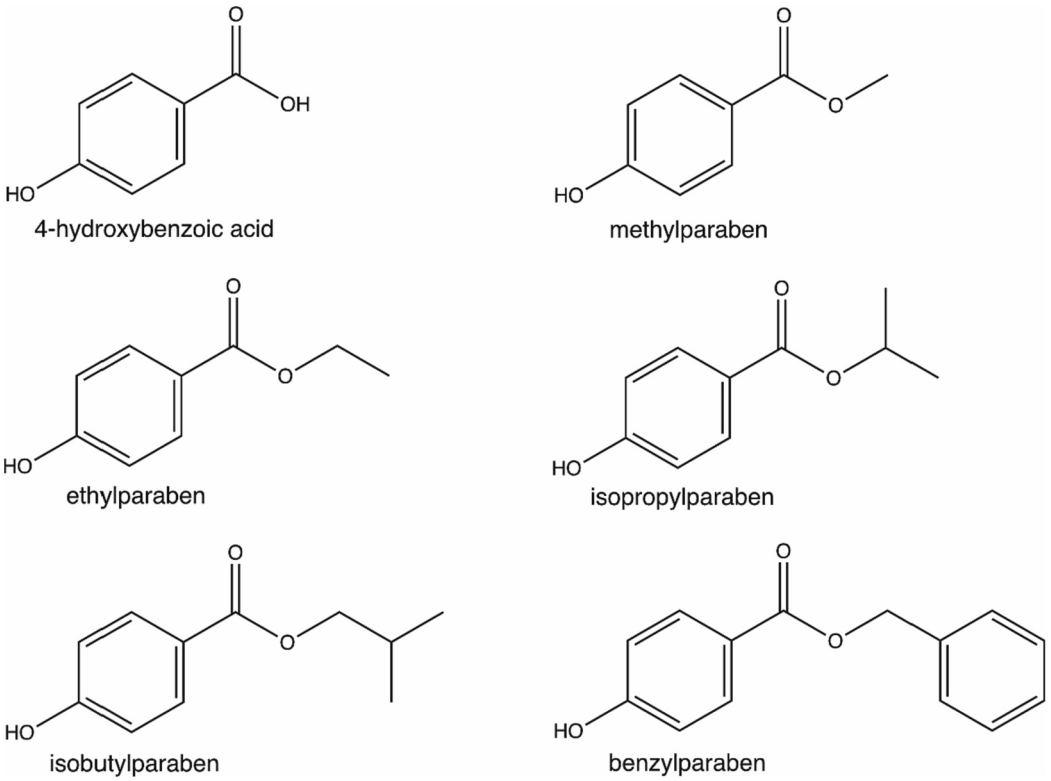


Fig. 1. Chemical structures of parabens covered by the study.

with flow rates of 1.0 mL min^{-1} (PC.MG and PC.DD2) and 0.3 mL min^{-1} (SPH). All samples were dissolved in ACN (*ca.* 10^{-4} M) and chromatographic analyses were carried out at 22 ± 2 °C. The eluents were obtained directly by mixing the acetonitrile and the aqueous buffer and the mixtures were subsequently degassed in an ultrasonic bath. All mobile phases were vacuum filtered through 0.22 μm nylon membranes (Millipore, Burlington, MA USA). Stock solutions of all PBs were prepared in ACN at 14–9 mM concentration range. Working solutions were freshly prepared daily by diluting the stock solutions in ACN at 72–22 μM final concentration range, filtered through 0.22 μm membrane and a 20 μL sample was injected into the LC system. Sample solutions were stored in the freezer at −18 °C and were used for no more than 90 days. The working solutions were discarded at the end of each day. The affinity for all five stationary phases was determined as chromatographic retention coefficient k, defined as:

$$k = \frac{t_r - t_0}{t_0} \quad (1)$$

where, t_r and t_0 are the retention times of the analyte and a non-retained compound (acetone), respectively, and reported as logarithm, log k. The affinity values refer to a 100 % (*v*/v) aqueous eluent and are achieved by an extrapolation method (Braumann et al., 1983) varying the ACN in various percentages (φ), due to both protein and phospholipid based stationary phases requiring organic modifier to allow elution of the target analytes.

2.3. ADMET predictor calculations

PBs toxicity was predicted using Simulations-Plus ADMET Predictor™ Version 7.1 (Simulation Plus, Lancaster, CA, USA) for Windows-based personal computers, a program able to estimate properties such as Absorption, Distribution, Metabolism, Excretion, and Toxicity starting from the molecular structure of the compounds with a high degree of accuracy. The software uses artificial neural network ensemble (ANNE) models (Paixão et al., 2014) trained with well-defined drugs. A default pH 7.4 was chosen for *in silico* predictions and, concerning the ADMET data, percent unbound to plasma proteins, blood-to-plasma concentration ratio and volume of distribution were considered.

2.4. Cellular assays

2.4.1. Cell culture and treatment

MCF-7 cells were maintained aseptically, being split and passaged in RPMI medium consisting of 88.0 mL of RPMI 1640 medium, supplemented with 10 % (*v*/v) FBS, 1 % penicillin/streptomycin 1:1 10.0 mg/mL in a combined solution and 1 % of 200 mM L-glutamine. Drug treatment was carried out using reduced RPMI medium prepared as before, but with phenol red-free RPMI medium supplemented with 10 % charcoal stripped FBS, to avoid any external estrogenic influences due to the presence of phenol red (Berthois et al., 1986).

To optimise cell concentration for the study, cells were incubated for 48, 72, and 96 h in three 96-well plates without any drug treatment, at concentrations of 1×10^4, 5×10^4, 1×10^5 and 5×10^5 cells/mL. An MTT assay was then carried out to evaluate cell growth after incubation, and an optimal plating concentration of 1×10^4 cells/mL chosen.

For repetitions of the assay, after adjusting the cell concentration to 1×10^4 cells/mL, 150 μL of cell and medium solution was added to all wells of the 96-well plate, except for the blank sample (medium only). Cells were checked for growth and contamination a day later. 70 nM 17β-estradiol was used as a positive control,

DMSO at a concentration equivalent to the maximum amount used in PBs preparations was used as a solvent control, and cells treated with 0.1 % Triton X in PBS were used as a negative control, this being a detergent which induces cell membrane disruption and consequent cell death (Strupp et al., 2000).

The cells were then treated by addition of 50 μL of all controls and PBs to bring the final concentration within the wells to between 0.01 μM to 100 μM. The plate was then incubated for 72 h at 37 °C with a 5 % CO_2 humidified atmosphere with nine to eleven replicates undertaken for each PB concentration.

2.4.2. MTT assay

A fresh stock solution of 5.0 mg/mL MTT (Sigma-Aldrich UK Ltd.) was prepared in PBS and wrapped in tin foil to protect it from sunlight. Thereafter, a working MTT solution was made by adding 2.0 mL of stock MTT solution to 5.0 mL of treatment medium, for a total volume of 7.0 mL. Before adding MTT to the plate, 50.0 μL of Triton was added to the negative control wells, and the plate incubated again for few minutes and then removed. All previous medium present in the wells was then removed and replaced with fresh treatment medium and 50.0 μL of MTT solution. The plate was wrapped in tin foil and incubated for 3–4 h at 37 °C. After incubation, all medium was removed carefully from the wells and replaced with 150.0 μL of DMSO to dissolve the blue crystals formed during the intervening reaction. Thereafter, the plate was kept in dark for 30 min before running on a Sunrise plate reader using Magellan Tecan software. This was carried out using an absorbance wavelength of 540 nm and a reference wavelength of 620 nm.

2.4.3. Statistical analysis

Data analyses were performed by a 2-way ANOVA (Minitab 21), Microsoft Excel (Office 365) and IBM SPSS Statistics 29.0.1.1 on a quad-core Windows 10 - based PC.

3. Results and discussion

3.1. Chromatographic analysis

As PBs are neutral compounds, their retention is mainly determined by hydrophobicity-based interactions, with polar/electrostatic forces playing an only minor role. Phospholipophilicity, *i.e.* the affinity for phospholipids (Russo et al., 2019; Russo et al., 2017) of the six chemicals was assessed by determining log k_w^{IAM} using IAM.PC.MG, IAM.PC.DD2 and IAM.SPH columns, either at pH 5.0 (to simulate the skin pH) or at pH 7.0 (to mirror the blood). Table 1 shows the results achieved by all chromatography stationary phases employed in this study. The IAM stationary phases consist of phosphatidylcholine analogues mimicking biological cell membrane (Russo et al., 2018; Russo et al., 2023). PC.DD2 differs from PC.MG in the end-capping of residual amino groups of the silica-propylamine core supporting C_{10} and C_3 alkyl chains being end-capped by both decanoic and propionic anhydrides, respectively while PC.MG supports hydroxy groups, being end-capped by methyl glycolate (Grumetto and Russo, 2021; Grumetto et al., 2015). IAM parameters performed on all stationary phases have been already found strongly interrelated for both pH values (pH 7.0:

Table 1
Logarithms of retention coefficients of PBs on HSA, AGP, IAM.PC.MG, IAM.PC.DD2 and IAM.SPH stationary phases. The values refer to a 100 % (*v*/v) aqueous eluent and are achieved by an extrapolation method. Fluctuations on the chromatographic retention factors never exceeded 4 % and are the averages of at least independent measurement per each % of organic modifier ($n = 4$), nr = not retained, nd = not detected.

Solute	pH 7.0					pH 5.0		
	log k_w^{HSA}	log k_w^{AGP}	log $k_w^{IAM.MG}$	log $k_w^{IAM.DD2}$	log $k_w^{IAM.SPH}$	log $k_w^{IAM.MG}$	log $k_w^{IAM.DD2}$	log $k_w^{IAM.SPH}$
pHBA	nr	nr	−0.96	−1.05	−0.49	nr	0.41	0.51
MP	1.18	1.03	0.61	1.31	1.08	0.63	1.07	1.09
EP	1.16	1.49	0.96	1.75	1.46	0.99	1.55	1.49
iPrP	1.29	0.92	1.23	1.91	1.82	1.29	1.89	1.89
iBuP	1.71	1.19	1.71	2.45	2.35	1.76	2.62	2.77
BzP	nd	nd	2.29	2.73	2.72	2.14	3.08	2.78

log $k_w^{PC.MG}$ *vs* log $k_w^{PC.DD2}$, $y = 1.1945\ (\pm 0.1134)x + 0.3540\ (\pm 0.1597)$, $r^2 = 0.965$; pH 5.0: log $k_w^{PC.MG}$ *vs* log $k_w^{PC.DD2}$, $y = 1.3465\ (\pm 0.0345)x + 0.2081\ (\pm 0.0505)$, $r^2 = 0.998$) as assessed in our previous studies (Grumetto et al., 2012; Grumetto et al., 2016b; Russo et al., 2017). This is not surprising as it was demonstrated that the different end capping has a marginal role in the affinity between chemicals and the IAM phase and that, in IAM LC, the analytical retention of neutral molecules mainly depends on their *n*-octanol/water partitioning (Grumetto et al., 2016a; Tsopelas et al., 2016). Consistently, BzP, which is the solute with the highest molecular mass (228.24 Da) is the compound retained for the longest time on all the IAM phases.

The binding to serum proteins determines the available concentration of toxicants. Indeed, binding to the HSA and AGP can occur at many structural and functional subdomains on this protein *via* forces of both electrostatic and hydrophobic nature. However, the two PP biomimetic columns exhibit a different selectivity particularly with electrolytes, as the HSA affords a stronger retention of anions whereas the AGP typically retains cations as compared to a hypothetical neutral isolipophilic molecule. According to our HSA and AGP LC experiments, the highest affinity of this plasma protein was observed for iPrP and iBuP as affinity of BzP for these PP could not be measured due to excessive retention.

3.2. In vitro bioscreenings for cell response

All the studied compounds were found to induce proliferation of MCF-7 cells; however, this was more marked for those having longer and branched alkyl side chains compared to those with short and straight chains. The proliferation of MCF-7 cells, epithelial-like type human breast adenocarcinoma cells, was estimated after treatment of cells with PBs, pHBA and E2 as positive control, to test their ability to induce cell growth (Fortunati et al., 1999). E2, at a concentration of 70 nM, induced cell proliferation to an extent 130 % greater than untreated cells. Amongst the six chemicals under investigation, those having longer/branched side chain showed higher proliferation rates in MCF-7 cells, and in particular, iPrP at 1.0 μM induced a proliferation that was about 20 % greater than E2 (150% *vs* untreated cell). In comparison, the other PBs, BzP and iBuP, at concentrations of 10.0 and 0.1 μM respectively, caused cell growth lower than E2, while MP and EP had a slightly proliferative effect on MCF-7, as can be seen in Fig. 2. A possible explanation of this behaviour is that sterically hindered esters undergo hydrolysis to a lower extent than linear, less bulky esters (Xu et al., 2021). Statistical analysis carried out (2-way ANOVA) indicated that there was significance with respect to the effects of the different drugs ($p < 0.001$), but that there was no significant relationship with concentration (therefore concentration-independent), and no relationship for a combination of drug and concentration.

3.3. Relationships between biomimetic LC and cell data

We employed several biomimetic stationary phases *i.e.*, HSA, AGP,

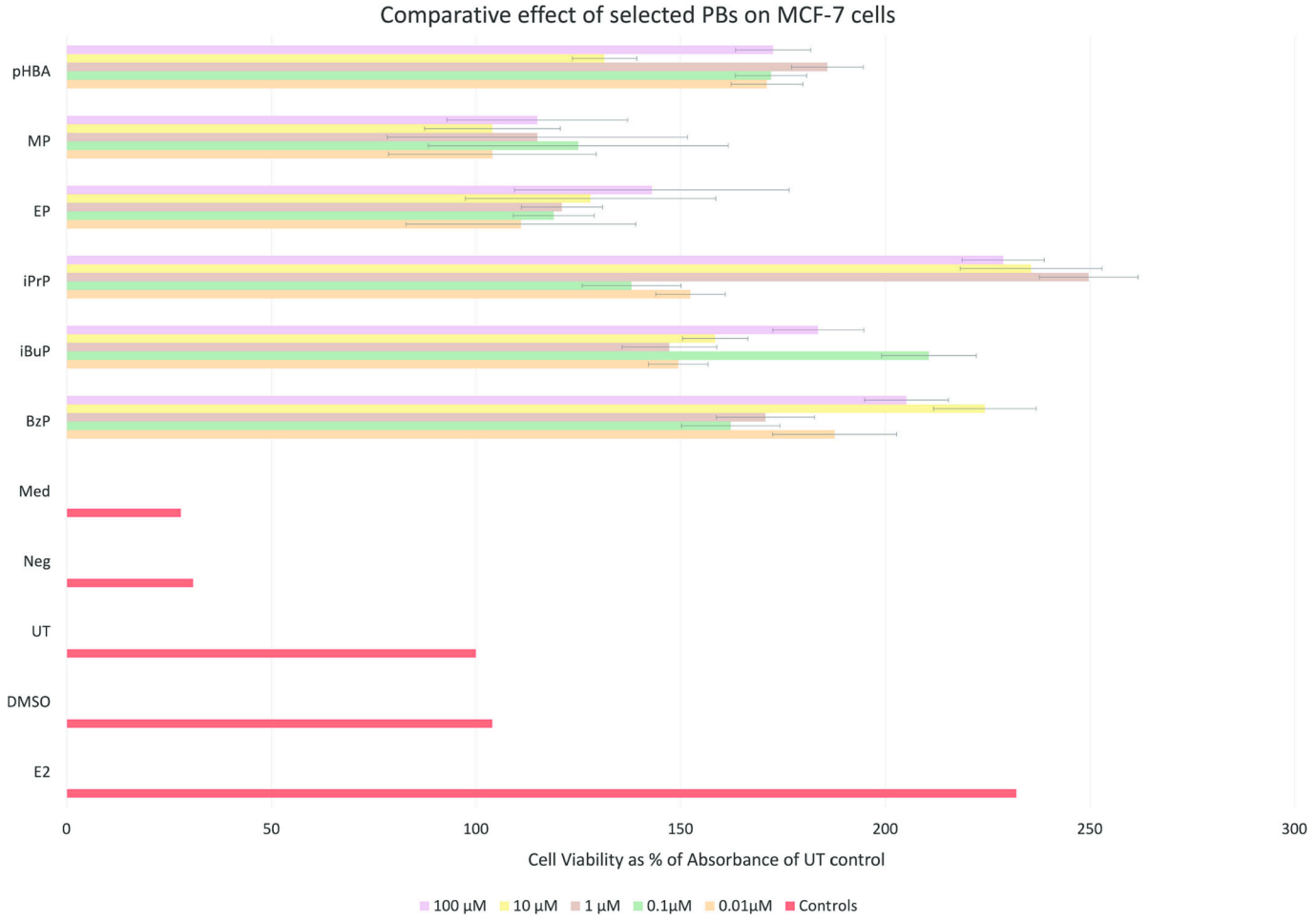


Fig. 2. Plot showing the effect of the selected compounds in terms of cell viability as % of Absorbance of untreated cell (UT) control. Med – Medium only control; Neg – triton-X treated cells; DMSO – DMSO control; E2 17β estradiol70nM; pHBA – p-hydroxybenzoic acid; MP – Methyl 4-hydroxybenzoate; EP – Ethyl 4-hydroxybenzoate; iPrP – Isopropyl 4-hydroxybenzoate; iBuP – Isobutyl 4-hydroxybenzoate; Bz – Benzyl 4-hydroxybenzoate. All data points represent mean and standard deviation for 9–11 replicates. 2-way ANOVA indicated significant differences in the responses to different parabens ($p < 0.001$) but not to concentration, and not to concentration and chemical combined.

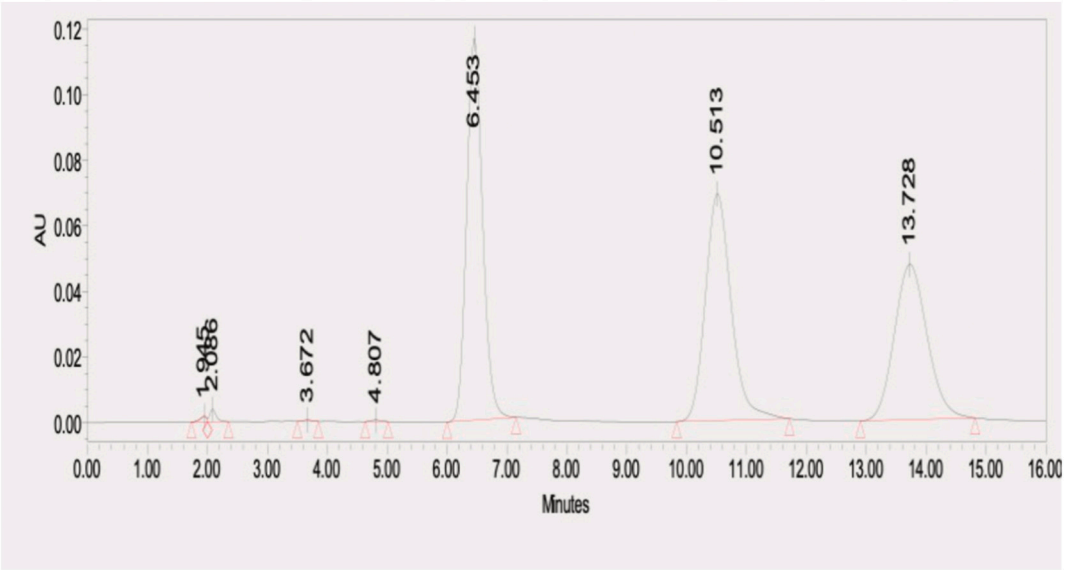


Fig. 3. HPLC chromatogram; from left to right, iPrP, iBuP, BzP in 70/30 0.1 M KH_2PO_4 buffer pH 7/ACN. Bz – Benzyl Paraben; iBuP – IsoButyl Paraben; iPrP – IsoPropyl Paraben. Experimental conditions are detailed in 2.2.

IAM.PC.MG, IAM.PC.DD2 and IAM.SPH stationary phases, to explore possible relationships between the PBs affinity for biological structures and proliferative effect in MCF-7 cells. The differences between readout and control on the MCF-7 cell line (delta viability) at all the assayed concentrations of parabens are reported in Table S1. The retention factors of only four solutes out of six could be determined by HPLC on the PP HSA and AGP, therefore the conclusions that we can draw are limited. The relationship between log k_w^{HSA} and the difference in proliferative effect between the readout and the control at 0.1 μM was very good and is reported in the Fig. S1. Other than that, all the other relationships between PP biomimetic LC and delta viability were very weak. LC approach performed on an IAM stationary phases, allows high reproducibility, yielding log k_w^{IAM} values as a direct measure of the phospholipophilicity, *i.e.*, the analyte affinity for phosphatidylcholine, as well as sphingomyelin, typically found in the membranous myelin sheath that surrounds some nerve cell axons (Bieberich, 2018). An exemplative chromatogram is shown in Fig. 3. The best relationship between the difference in proliferative effect between the readout and the control was at 0.01 μM concentration and the retention on the IAM phases MG ($r^2 = 0.893$) and SPH ($r^2 = 0.887$) measured at pH 7.0 was observed for all compounds but pHBA, which is the only analyte of the dataset supporting a carboxylic acid moiety. However, pHBA is the only carboxylic acid, so it is structurally unrelated with the other molecules, thus the interaction with the biological target (Fig. 4A and B) may be based on structural moieties that are not supported by other solutes in the dataset.

The present findings should be cautiously considered. One limitation of the experimental design is that cell viability was assessed only on a cancerous cell line *e.g.*, MCF-7, but not on the normal counterpart *e.g.* MCF-10a, this implies that the effects are not necessarily relatable to a healthy tissue. Moreover, for some extremely polar and extremely hydrophobic molecules *e.g.*, pHBA and BzP, chromatographic affinity on PPs could not be determined. This depends on the aspect that pHBA was not retained at all by protein stationary phase whereas BzP could not be eluted using eluents containing 20 % 2-propanol (*v*/v). Indeed, according to the manufacturer's guidelines, using organic modifier at higher concentration may result in the denaturation of the protein with consequential loss in reproducibility and retention (Fournier et al., 2000).

3.4. Relationships between in silico calculated properties, cell proliferation, and phospholipophilicity

In the last years, ADMET Predictor™ software (Ghosh et al., 2016) has become a well-recognized tool for its role in predicting properties, nevertheless it is important that the employment of *in silico* and chromatographic data is supported by *in vitro* cell experiment. Permeability of a molecule, for oral/topical administration, depends on the extent of their transportation through the walls of cells. Concerning PBs, it can provide usefulness in predicting the permeability rate as a crucial parameter for the estimation of cell crossing (Neri et al., 2022). We found an excellent correlation ($r^2 = 0.982$, significant at the 0.01 level (2-tailed)) between affinity data achieved on PC.DD2 at pH 7.0 and P_{eff}, the predicted human effective jejunal permeation, supporting the evidence that the more these compounds interact with the phospholipid-like phase, the more these can cross the biomembranes. In fact, it has been reported that chemicals with a P_{eff} value > 1.5 are completely absorbed no matter which transport mechanism(s) is utilized (Lennernäs, 2007). Fig. 5 shows that the P_{eff} values for all the PBs are >1.5, suggesting completed absorption. Our analytes showed values well above 1.5 (>3.0 for pHBA and above 4.0 for other compounds), indeed they were considered as highly permeable compounds, therefore growing in toxicity scale. Furthermore, *in silico* calculated Log BB, considered as an indicator for Blood Brain Barrier delivery, was also found related to data achieved on PC.DD2 at pH 7.0 ($r^2 = 0.914$, significant at the 0.01 level (2-tailed)), according to literature data concerning PBs penetration into the brain (Denuzière and Ghersi-Egea, 2022). Diffcoeff, an Hayduk-Laudie infinite dilution diffusion coefficient ($cm^2/s^* 105$), was found to be inversely related with all the chromatographic data, particularly for IAM data achieved on both PC.MG and PC.DD2 stationary phases ($r^2 = 0.976$, significant at the 0.01 level (2-tailed)). This is not surprising as according to the Stokes–Einstein equation, the diffusion coefficient of a molecule is expected to increase in inverse proportion to its approximate radius (Dubey et al., 2021). Therefore, a larger molecular weight is related to a larger approximate radius, so that the diffusion coefficient is generally smaller. For neutral molecules, retention on the biomimetic stationary phases depends upon hydrophobicity interactions, given that no electrostatic contribution can be reasonably observed. Hydrophobicity is directly related to the molecular volume of the chemical, which is again linearly dependent on the approximate radius. These results are consistent with those achieved by

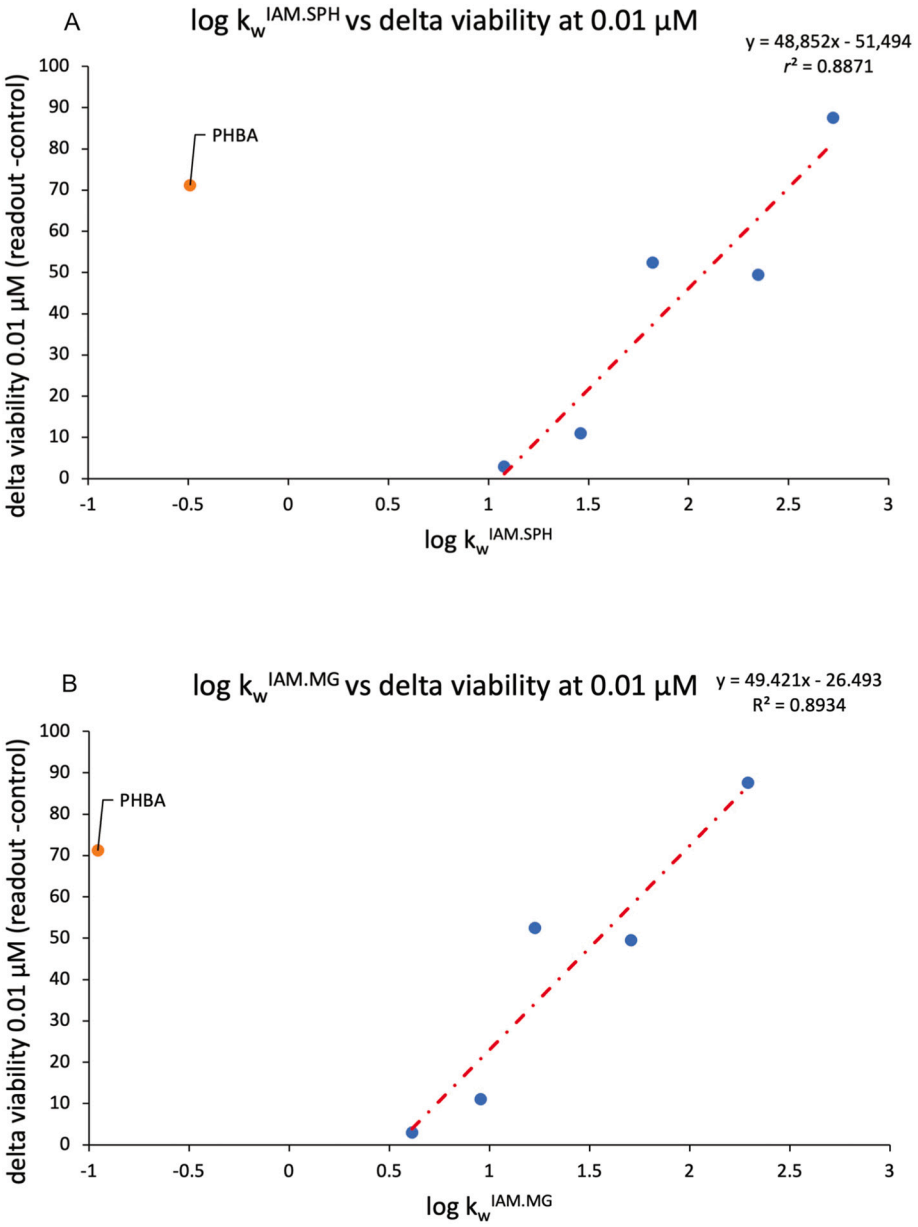


Fig. 4. Relationships between readout and control on the MCF-7 cells and log k_w^{IAM} on both (A) sphingomyelin (SPH) ($q^2 = 0.86$, SE = 0.25) and (B) MG ($q^2 = 0.85$, SE = 0.26) phases achieved at pH 7.0.

Okubo et al. (2001) who studied the estrogenic activities of MP, EP, propylparaben, butylparaben, iPrP and iBuP by assaying estrogen-receptor (ER)-dependent proliferation of MCF-7 cells. They concluded that the parabens with longer sidechains showed greater affinity for estrogen receptors and that these exhibited similar relative binding affinity values to both ERα and ERβ. Our results also agree with those by Charles and Darbre (2013) who studied the proliferation effects on MCF-7 of both individual parabens and their combinations. Specifically, the authors determined both EC_{50} and EC_{100}, the lowest concentrations for half maximal – and maximal – simulation of proliferation, respectively, of a range of PBs which included MP, EP and iBuP. Both these ranked in the order MP (10^{-4} M) > EP (10^{-5} M) > iBuP (10^{-6} M), suggesting that iBuP is the PB able to induce cell proliferation at lower concentration. It is noteworthy to indicate that comparison with the raw numerical data is not appropriate, given that the experimental conditions were different as the ECs were assessed after 7 and 14 days in culture which is longer than in the present study. Elsehly et al. (2022), instead, evidenced a proliferative effect on MCF-7 cell of MP that was concentration dependent, however this does not contradict any of our findings as this effect was visible at concentrations that are much higher (40–800 µM) than ours (0.01–100 µM). Indeed, if we look at the work by Wróbel and Gregoraszczuk (2013), who assessed the proliferation and oestradiol secretion on MCF-7 cells, the concentration dependency of MP on cell viability was lost in the 0.2 nM–2 µM range, which partially overlaps ours. Clearly, cell proliferation is not the only hallmark of cancer cells, and in this context the study by Khanna et al. (2014) is particularly interesting as it covers the migratory and invasive activity of breast cancer cells *in vitro* upon exposure to PBs. The authors reported that the rate of cell invasion through matrigel-coated membranes was increased most markedly after long-term exposure to MP as compared to other longer chained PBs, however again the concentration applied to study the invasive properties are one order of magnitude higher for MP (10^{-4} M) as compared to the other PBs assayed (10^{-5} M). Moreover, the use of predictive toxicology tools to assess the endocrine-disrupting potential of parabens *in silico* has been recently proposed by both Jakopin (2021) and Smith et al. (2020). This approach is based on calculating the

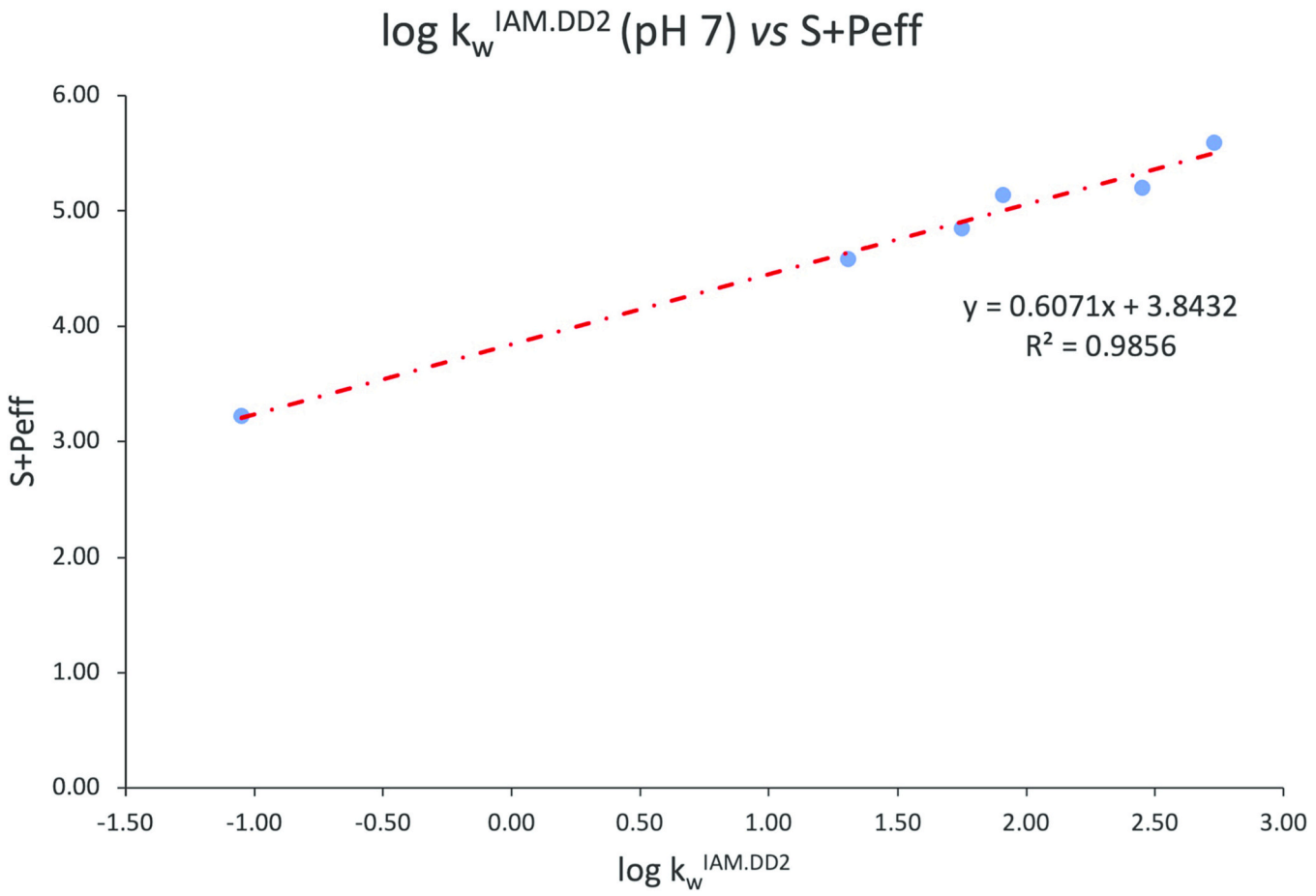


Fig. 5. Relationship between effective permeability ($S + P_{eff}$) and Immobilized Artificial Membrane (IAM) affinity data on the IAM.DD2 phase at pH 7.0 ($q^2 = 0.98$, SE = 0.11).

binding affinities of PBs to an array of nuclear receptors. All the output of ADMET Predictor™ software is available as supplementary material (Table S2).

4. Conclusion

The potential of biochromatography to predict PBs affinity for biological structures was evaluated along with cell proliferation activity. IAM LC has historically been exploited to estimate the absorption potential of therapeutics. The technique can offer effectiveness in profiling the toxicity of compounds typically belonging to the same class and is particularly discriminative when the toxicity depends mainly upon the toxicokinetic of the chosen chemicals but not or not noticeably on their toxicodynamic. Biomimetic chromatography is robust in simulating interactions between xenobiotics and biological structures both using immobilized artificial membranes and proteins. Our chromatographic data relate quite well to conventional *in vitro* methods such as MCF-7 cell proliferation assay, suggesting that toxicity can be mediated by a higher potential of passage through biological membranes. The study shows that biochromatography may be a valuable experimental tool in predicting cellular permeability and biodistribution process of xenobiotics, and therefore, their possible toxicity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170461.

CRediT authorship contribution statement

Ilaria Neri: Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Janis MacCallum:** Writing – original draft, Validation, Methodology, Investigation, Data curation. **Ritamaria Di Lorenzo:** Data curation. **Giacomo Russo:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Frédéric Lynen:** Writing – review & editing. **Lucia Grumetto:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The MSc student Eloise Debret is gratefully acknowledged for contributing to the data generation and article graphics.

References

Abbas, S., Greige-Gerges, H., Karam, N., Piet, M.H., Netter, P., Magdalou, J., 2010. Metabolism of parabens (4-hydroxy-benzoic acid esters) by hepatic esterases and UDP-glucuronosyltransferases in man. Drug Metab. Pharmacokinet. 25, 568–577.

Berthois, Y., Katzenellenbogen, J.A., Katzenellenbogen, B.S., 1986. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc. Natl. Acad. Sci. U. S. A. 83, 2496–2500.

Bieberich, E., 2018. Sphingolipids and lipid rafts: novel concepts and methods of analysis. Chem. Phys. Lipids 216, 114–131.

Braumann, T., Weber, G., Grimme, L.H., 1983. Quantitative structure—activity relationships for herbicides: reversed-phase liquid chromatographic retention parameter, log kw, versus liquid-liquid partition coefficient as a model of the hydrophobicity of phenylureas, s-triazines and phenoxycarbonic acid derivatives. J. Chromatogr. A 261, 329–343.
Byford, J.R., Shaw, L.E., Drew, M.G.B., Pope, G.S., Sauer, M.J., Darbre, P.D., 2002. Oestrogenic activity of parabens in MCF7 human breast cancer cells. J. Steroid Biochem. Mol. Biol. 80, 49–60.
Carson, R., 2002. Silent Spring. Houghton Mifflin Harcourt.
Charles, A.K., Darbre, P.D., 2013. Combinations of parabens at concentrations measured in human breast tissue can increase proliferation of MCF-7 human breast cancer cells. J. Appl. Toxicol. 33, 390–398.
Dagher, Z., Borgie, M., Magdalou, J., Chahine, R., Greige-Gerges, H., 2012. p-Hydroxybenzoate esters metabolism in MCF7 breast cancer cells. Food Chem. Toxicol. 50, 4109–4114.
Denuzière, A., Ghersi-Egea, J.-F., 2022. Cerebral concentration and toxicity of endocrine disrupting chemicals: the implication of blood-brain interfaces. NeuroToxicology 91, 100–118.
Dubey, V., Dueby, S., Daschakraborty, S., 2021. Breakdown of the Stokes-Einstein relation in supercooled water: the jump-diffusion perspective. Phys. Chem. Chem. Phys. 23, 19964–19986.
Elsehly, W.M., Mourad, G.M., Mehanna, R.A., Kholief, M.A., El-Nikhely, N.A., Awaad, A. K., et al., 2022. The potential implications of estrogenic and antioxidant-dependent activities of high doses of methyl paraben on MCF7 breast cancer cells. J. Biochem. Mol. Toxicol. 36, e23012.
Fanali, G., di Masi, A., Trezza, V., Marino, M., Fasano, M., Ascenzi, P., 2012. Human serum albumin: from bench to bedside. Mol. Aspects Med. 33, 209–290.
Fortunati, N., Becchis, M., Catalano, M.G., Comba, A., Ferrera, P., Raineri, M., et al., 1999. Sex hormone-binding globulin, its membrane receptor, and breast cancer: a new approach to the modulation of estradiol action in neoplastic cells. J. Steroid Biochem. Mol. Biol. 69, 473–479.
Fournier, T., Medjoubi, N.N., Porquet, D., 2000. Alpha-1-acid glycoprotein. Biochim. Biophys. Acta 1482, 157–171.
Ghosh, J., Lawless, M.S., Waldman, M., Gombar, V., Fraczkiewicz, R., 2016. Modeling ADMET. Methods Mol. Biol. 1425, 63–83.
Gomes, F.E., Bergo, P.L., Trap, M.A., Spadoto, M., Galinaro, C.A., Rodrigues-Filho, E., et al., 2018. Photolysis of parabens using medium-pressure mercury lamps: toxicity effects in MCF7, Balb/c 3T3 cells and Cerio-daphnia dubia. Chemosphere 208, 325–334.
Grumetto, L., Russo, G., 2021. cΔlog k(w) (IAM): can we afford estimation of small molecules' blood-brain barrier passage based upon in silico phospholipophilicity? Admet dmpk 9, 267–281.
Grumetto, L., Carpentiero, C., Barbato, F., 2012. Lipophilic and electrostatic forces encoded in IAM-HPLC indexes of basic drugs: their role in membrane partition and their relationships with BBB passage data. Eur. J. Pharm. Sci. 45, 685–692.
Grumetto, L., Russo, G., Barbato, F., 2015. Relationships between human intestinal absorption and polar interactions drug/phospholipids estimated by IAM-HPLC. Int. J. Pharm. 489, 186–194.
Grumetto, L., Russo, G., Barbato, F., 2016a. Immobilized artificial membrane HPLC derived parameters vs PAMPA-BBB data in estimating in situ measured blood-brain barrier permeation of drugs. Mol. Pharm. 13, 2808–2816.
Grumetto, L., Russo, G., Barbato, F., 2016b. Polar interactions drug/phospholipids estimated by IAM-HPLC vs cultured cell line passage data: their relationships and comparison of their effectiveness in predicting drug human intestinal absorption. Int. J. Pharm. 500, 275–290.
Hager, E., Chen, J., Zhao, L., 2022. Minireview: parabens exposure and breast cancer. Int. J. Environ. Res. Public Health 19.
Harvey, P.W., Everett, D.J., 2012. Parabens detection in different zones of the human breast: consideration of source and implications of findings. J. Appl. Toxicol. 32, 305–309.
Jakopin, Ž., 2021. Assessment of the endocrine-disrupting potential of halogenated parabens: an in silico approach. Chemosphere 264, 128447.
Janjua, N.R., Frederiksen, H., Skakkebaek, N.E., Wulf, H.C., Andersson, A.M., 2008. Urinary excretion of phthalates and paraben after repeated whole-body topical application in humans. Int. J. Androl. 31, 118–130.
Khanna, S., Dash, P.R., Darbre, P.D., 2014. Exposure to parabens at the concentration of maximal proliferative response increases migratory and invasive activity of human breast cancer cells in vitro. J. Appl. Toxicol. 34, 1051–1059.
Lee, J., Bang, S.H., Kim, Y.H., Min, J., 2018. Toxicities of four parabens and their mixtures to Daphnia magna and Aliivibrio fischeri. Environ. Health Toxicol. 33, e2018018.
Lennernäs, H., 2007. Intestinal permeability and its relevance for absorption and elimination. Xenobiotica 37, 1015–1051.
Moos, R.K., Angerer, J., Dierkes, G., Brüning, T., Koch, H.M., 2016. Metabolism and elimination of methyl, iso- and n-butyl paraben in human urine after single oral dosage. Arch. Toxicol. 90, 2699–2709.
Neri, I., Laneri, S., Di Lorenzo, R., Dini, I., Russo, G., Grumetto, L., 2022. Parabens permeation through biological membranes: a comparative study using Franz cell diffusion system and biomimetic liquid chromatography. Molecules 27.
Niede, R., Benbi, D.K., 2022. Integrated review of the nexus between toxic elements in the environment and human health. AIMS Public Health 9, 758–789.
Nowak, K., Ratajczak-Wrona, W., Górska, M., Jabłońska, E., 2018. Parabens and their effects on the endocrine system. Mol. Cell. Endocrinol. 474, 238–251.
Okubo, T., Yokoyama, Y., Kano, K., Kano, I., 2001. ER-dependent estrogenic activity of parabens assessed by proliferation of human breast cancer MCF-7 cells and expression of ERα and PR. Food Chem. Toxicol. 39, 1225–1232.
Paixão, P., Aniceto, N., Gouveia, L.F., Morais, J.A., 2014. Prediction of drug distribution in rat and humans using an artificial neural networks ensemble and a PBPK model. Pharm. Res. 31, 3313–3322.
Pena-Pereira, F., Bendicho, C., Pavlović, D.M., Martín-Esteban, A., Díaz-Álvarez, M., Pan, Y., et al., 2021. Miniaturized analytical methods for determination of environmental contaminants of emerging concern – a review. Anal. Chim. Acta 1158, 238108.
Petric, Z., Ružić, J., Žuntar, I., 2021. The controversies of parabens - an overview nowadays. Acta Pharm. 71, 17–32.
Russo, G., Grumetto, L., Barbato, F., Vistoli, G., Pedretti, A., 2017. Prediction and mechanism elucidation of analyte retention on phospholipid stationary phases (IAM-HPLC) by in silico calculated physico-chemical descriptors. Eur. J. Pharm. Sci. 99, 173–184.
Russo, G., Capuozzo, A., Barbato, F., Irace, C., Santamaria, R., Grumetto, L., 2018. Cytotoxicity of seven bisphenol analogues compared to bisphenol A and relationships with membrane affinity data. Chemosphere 201, 432–440.
Russo, G., Barbato, F., Grumetto, L., Philippe, L., Lynen, F., Goetz, G.H., 2019. Entry of therapeutics into the brain: influence of exposed polarity calculated in silico and measured in vitro by supercritical fluid chromatography. Int. J. Pharm. 560, 294–305.
Russo, G., Ermondi, G., Caron, G., Verzele, D., Lynen, F., 2021. Into the first biomimetic sphingomyelin stationary phase: suitability in drugs' biopharmaceutic profiling and block relevance analysis of selectivity. Eur. J. Pharm. Sci. 156, 105585.
Russo, G., Piccolo, M., Neri, I., Ferraro, M.G., Santamaria, R., Grumetto, L., 2023. Lipophilicity profiling and cell viability assessment of a selected panel of endocrine disruptors. Chemosphere 313, 137569.
Shin, M.Y., Shin, C., Choi, J.W., Lee, J., Lee, S., Kim, S., 2019. Pharmacokinetic profile of propyl paraben in humans after oral administration. Environ. Int. 130, 104917.
Smith, M.N., Cohen Hubal, E.A., Faustman, E.M., 2020. A case study on the utility of predictive toxicology tools in alternatives assessments for hazardous chemicals in children's consumer products. J. Expo. Sci. Environ. Epidemiol. 30, 160–170.
Strupp, W., Weidinger, G., Scheller, C., Ehret, R., Ohnimus, H., Girschick, H., et al., 2000. Treatment of cells with detergent activates caspases and induces apoptotic cell death. J. Membr. Biol. 175, 181–189.
Torfs, E., Brackman, G., 2021. A perspective on the safety of parabens as preservatives in wound care products. Int. Wound J. 18, 221–232.
Tsopelas, F., Vallianatou, T., Tsantili-Kakoulidou, A., 2016. Advances in immobilized artificial membrane (IAM) chromatography for novel drug discovery. Expert Opin. Drug Discovery 11, 473–488.
Verzele, D., Lynen, F., De Vrieze, M., Wright, A.G., Hanna-Brown, M., Sandra, P., 2012. Development of the first sphingomyelin biomimetic stationary phase for immobilized artificial membrane (IAM) chromatography. Chem. Commun. (Camb.) 48, 1162–1164.
Watanabe, Y., Kojima, H., Takeuchi, S., Uramaru, N., Ohta, S., Kitamura, S., 2013. Comparative study on transcriptional activity of 17 parabens mediated by estrogen receptor α and β and androgen receptor. Food Chem. Toxicol. 57, 227–234.
Wei, F., Mortimer, M., Cheng, H., Sang, N., Guo, L.H., 2021. Parabens as chemicals of emerging concern in the environment and humans: a review. Sci. Total Environ. 778, 146150.
Wróbel, A., Gregoraszczuk, E., 2013. Effects of single and repeated in vitro exposure of three forms of parabens, methyl-, butyl- and propylparabens on the proliferation and estradiol secretion in MCF-7 and MCF-10A cells. Pharmacol. Rep. 65, 484–493.
Xu, T., Chen, J., Chen, X., Xie, H., Wang, Z., Xia, D., et al., 2021. Prediction models on pK(a) and base-catalyzed hydrolysis kinetics of parabens: experimental and quantum chemical studies. Environ. Sci. Technol. 55, 6022–6031.
Yadav, D., Rangabhashiyam, S., Verma, P., Singh, P., Devi, P., Kumar, P., et al., 2021. Environmental and health impacts of contaminants of emerging concerns: recent treatment challenges and approaches. Chemosphere 272, 129492.