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Cellular accumulation and lipid binding of perfluorinated alkylated substances (PFASs) – A comparison with lysosomotropic drugs

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ABSTRACT

Many chemicals accumulate in organisms through a variety of different mechanisms. Cationic amphiphilic drugs (CADs) accumulate in lysosomes and bind to membranes causing phospholipidosis, whereas many lipophilic chemicals target adipose tissue. Perfluoroalkyl substances (PFASs) are widely used as surfactants, but many of them are highly bioaccumulating and persistent in the environment, making them notorious environmental toxicants. Understanding the mechanisms of their bioaccumulation is, therefore, important for their regulation and substitution with new, less harmful chemicals.

We compared the highly bioaccumulative perfluorooctanesulfonic acid PFOS to its three less bioaccumulative alternatives perfluorooctanoic acid (PFOA), perfluorohexanoic acid (PFHxA) and perfluorobutane sulfonic acid (PFBS), in their ability to accumulate and remain in lung epithelial cells (NCI-H292) and adipocytes (3T3-L1K) in vitro. As a reference point we tested a set of cationic amphiphilic drugs (CADs), known to highly accumulate in cells and strongly bind to phospholipids, together with their respective non-CAD controls. Finally, all compounds were examined for their ability to bind to neutral lipids and phospholipids in cell-free systems.

Cellular accumulation and retention of the test compounds were highly correlated between the lung epithelial cells and adipocytes. Interestingly, although an anion itself, intensities of PFOS accumulation and retention in cells were comparable to those of CAD compounds, but PFOS failed to induce phospholipidosis or alter lysosomal volume.

Compared to other lipophilicity measures, phospholipophilicity shows the highest correlation (R^2 = 0.75) to cellular accumulation data in both cell types and best distinguishes between high and low accumulating compounds. This indicates that binding to phospholipids may be the most important component in driving high cellular accumulation in lung epithelial cells, as well as in adipocytes, and for both CADs and bioaccumulating PFASs. Obtained continuous PLS models based on compound's affinity for phospholipids and neutral lipids can be used as good prediction models of cellular accumulation and retention of PFASs and CADs.

1. Introduction

A large number of compounds display some bioaccumulative behavior, but the mechanisms of their accumulation in organisms may differ. Persistent Organic Pollutants (POPs) are organic chemicals which, once released into the environment, remain unchanged for many years, accumulate in living organisms and have toxic effects on humans and wildlife. The United Nations Environment Programme (UNEP) made the decision to strictly regulate POPs in 2001 via The Stockholm Convention (active since 2004) [1]. Understanding the mechanisms behind bioaccumulation and finding methods to screen for it is crucial for the risk assessment of these chemicals.

Due to their high lipophilicity, POPs mainly accumulate in adipose tissue, and this characteristic is predictable from LogP ($LogK_{OW}$), the

Abbreviations: ACC, compound's accumulation in cells; ACE, acetaminophen; ACN, acetonitrile; AMIO, amiodarone; AMIT, amitriptyline; AZI, azithromycin; AZAG, azithromycin-aglycon; CAD, cationic amphiphilic drug; CHIIAM7.4, chromatographic index of immobilized artificial membrane at pH7.4; CHL, chloroquine; DMSO, dimethylsulphoxide; ERY, erythromycin; FBS, fetal bovine serum; FLU, fluoxetine; IAM, immobilized artificial membrane; IMI, imipramine; IND, indomethacin; LogD7.4, distribution coeficient of total charged and non-charged species of compound between octanol and water at pH7.4; LogP, $LogK_{OW}$; distribution coeficient of non-charged species of compound between octanol and water, NH_4Ac; ammonium acetate, OFL; ofloxacin, PFASs; perfluoroalkyl substances, perfluorinated alkylated substances; PFBS, perfluorobutane sulfonic acid; PFHxA, perfluorohexanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; POP, persistent organic pollutant; RET, compound's retention in cells; SD, standard deviation; TP, total cellular proteins

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partition coefficient of a molecule between lipophilic (octanol) and water phases. However, some POPs belonging to perfluoroalkyl substances (PFASs), bioaccumulate in a different manner despite their lipophilicity. Perfluorooctane sulfonic acid (PFOS) has been listed in the Annex B (Compounds with restricted production and use) of The Stockholm Convention, and was reported to accumulate in organisms mainly due to its binding to plasma proteins, as well as sequestration into the liver, kidney and lungs [2,3]. The half-life of PFOS in humans has been estimated to greater than five years [4]. Perfluorooctanoic acid (PFOA), known for being present in polytetrafluoroethylene (PTFE)-coated cookware (such as Teflon™) is also bioaccumulative, with a half-life in humans of more than three years [4]. PFOA, and the less bioaccumulative perfluorohexanoic acid (PFHxA) [5], are currently under review to be listed as POPs. Shorter chain derivative, such as perfluorobutane sulfonic acid (PFBS), with the half-life of a couple of weeks in humans [6], is not considered bioaccumulative and is often suggested as replacements for longer chain PFASs.

Perfluorinated compounds are mainly used as surfactants, in fire extinguishers and as water repellents in the textile industry. More than two hundred derivatives currently exist in the environment, emitted either from chemical industry or as metabolites and degradation products of synthesized forms [7]. Since many have bioaccumulative and persistent properties, they pose a great threat to ecosystems and human health, and understanding the mechanisms of their accumulation, as well as finding ways to predict this for a large number of compounds, is of great importance for the risk assessment and regulation of this class of POPs.

As another example of bioaccumulative compounds, cationic amphiphilic drugs (CADs) accumulate in acidic compartments of cells, such as lysosomes, a trait known as lysosomotropism. As weak bases, CADs are protonated in acidic milieu of the lysosome, thus becoming impermeable for the lysosomal membrane, leading to trapping and concentration of the compound in the lysosome against the concentration gradient. Already after 3 h of exposure, such compounds can achieve up to 100–500 fold higher intracellular than extracellular concentration [8], leading to underestimation of exposures based on concentrations in serum or other cell-free biological fluids. Due to lysosomal trapping, CADs have a high affinity for tissues especially lung, liver or kidney [9]. Binding to phospholipids plays an important role in this type of accumulation and ultimately impairs the normal degradation of phospholipids leading to the accumulation of phospholipids in the cells – the condition known as phospholipidosis [10]. At least in the case of macrolide CADs, a positive correlation has been shown between a compound's ability to bind to phospholipids, its accumulation in cells and the induction of phospholipidosis [11].

In our previous work, we have analyzed the accumulation of macrolide CADs in a variety of human primary cell types of pulmonary and immune system origin [12]. There we reported that the relative ranking of macrocycles according to the intensity of accumulation in cells is similar across all cell types studied, indicating that lysosomal trapping, rather than some other cell-specific accumulation mechanisms, is most relevant for the overall accumulation of these compounds. The data obtained in various human primary cells highly correlated to data obtained on the NCI-H292 lung epithelial cell line. However, cell-specific expression of *P*-glycoprotein (MDR1), an ABC transporter with affinity for macrolides, may affect this generic behavior [13]. Still, in those studies we did not examine bioaccumulation in fat-loaded cells such as adipocytes. It is conceivable that due to their high lipid load, adipocytes may show different overall affinities for various CADs.

Therefore, the aim of this study was to measure the cellular accumulation and retention of selected PFASs covering a span of bioaccumulation properties from low to high and to investigate mechanisms behind it. To put PFASs intensities of accumulation in a better known context, they have been compared to a set of highly accumulating CADs and their respective non-CAD controls. Compound accumulation and retention in adipocytes has been compared to that in lung epithelial cells to investigate the role that neutral lipids may have in the accumulation of PFASs and CADs. Compound affinities for phospholipids and neutral lipids have been studied in cell-free systems to further clarify the mechanisms of accumulation. Finally, the data indicate a practical suit of screening and prediction models which can be used to predict the extent and main mechanisms of bioaccumulation of perfluorinated alkylated chemicals, which would be valuable for their regulation and use.

2. Materials and methods

2.1. Compounds

Acetaminophen (ACE), amiodarone hydrochloride (AMIO), amitriptyline hydrochloride (AMIT), chloroquine diphosphate (CHL), dimethylsulphoxide (DMSO), imipramine hydrochloride (IMI), indomethacin (IND), ofloxacin (OFL), erythromycin (ERY), fluoxetine (FLU), were purchased from Sigma-Aldrich, azithromycin-dihydrate (AZI) was from USP Standards, and azithromycin-aglycon (AZAG) was a kind gift from Fidelta. PFOS and PFBS were a kind gift from Dr. Johan Ericsson (Stockholm University), and PFOA and PFHxA were purchased from Sigma-Aldrich. All compounds were run on LC-MS and purity was estimated to more than 95%.

All test compounds were dissolved in DMSO at the concentration of 30 mM, except amiodarone (15 mM). Chloroquine was dissolved at 30 mM in deionized water (MilliQ, Millipore) and ofloxacin in 1 M NaOH.

Calibrator compounds used for phospholipid binding experiments included octanophenone, heptanophenone, hexanophenone, acetanilide, acetophenone and acetaminophen; and for lipid binding atenolol, sulpiride, metoprolol, labetalol, diltiazem, and triphenylene, and were all purchased from Sigma-Aldrich.

2.2. Cell culture

The human NCI-H292 lung epithelial mucoepidermoid carcinoma cell line, murine 3T3-L1K preadipocyte embryonic fibroblast cell line, murine RAW264.7 macrophage cell line and human HepG2 hepatocarcinoma cell line were all cultured according to ATCC protocols. Before the experiment, 3T3-L1K cells were differentiated into adipocytes according to [14] with slight changes.

Briefly, 3T3-L1K cells were seeded in 12-well plates 16000/well in 0.8 mL culture medium (DMEM with 10% bovine calf serum (BCS, Sigma)). When cells reached confluence (day 0 of differentiation) they were differentiated into adipocytes using differentiation medium: DMEM (Gibco) containing 4.5 g/L glucose, 1 mM sodium-pyruvate, 10% fetal bovine serum (FBS, Gibco), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma), 1 μg/mL insulin (Sigma) and 0.25 μM dexamethasone (Sigma) for 4 days with medium change on day 2. On day 4 of differentiation, the cells obtained the differentiation medium without IBMX and dexamethasone, and the medium was changed on day 7. On day 9 from the beginning of differentiation, lipid droplets were fully developed (as shown by BODIPY 493/503 staining), and the cells were used for experiments as adipocytes.

2.3. Cellular accumulation

Accumulation and retention experiments in NCI-H292 cells were conducted according to [12]; with the method being adapted for differentiated 3T3-L1K adipocytes. Briefly, cells in 12-well plates were exposed to 10 μM test compounds for 3 h in 1.2 mL of RPMI (NCI-H292 cells) or DMEM medium (for 3T3-L1K adipocytes). The cells were subsequently washed four times with cold phosphate buffered saline (PBS) and lysed by freezing to −20 °C in 0.3 mL 0.5% TritonX-100. To measure compound retention in cells after loading with test compounds, cells were washed and incubated in medium without test compounds for another 3 h and then washed and lysed.

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