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# Synthesis, physicochemical properties and *in vitro* cytotoxicity of nicotinic acid ester prodrugs intended for pulmonary delivery using perfluorooctyl bromide as vehicle

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**Abstracts**

This study explores perfluorooctyl bromide (PFOB) as a potential vehicle for the pulmonary delivery of a series of prodrugs of nicotinic acid using cell culture studies. The prodrugs investigated have PFOB–water (log $K_p$ = 0.78 to >2.2), perfluoromethylcyclohexane–toluene (log $K_p$ = −2.62 to 0.13) and octanol–water (log $K_p$ = 0.90–10.2) partition coefficients spanning several orders of magnitude. In confluent NCI-H358 human lung cancer cells, the toxicity of prodrugs administered in culture medium or PFOB depends on the medium of administration, with EC20's above 8 mM and 2.5 mM for culture medium and PFOB, respectively. Short-chain nicotinates administered both in PFOB and medium increase cellular NAD/NADP levels at 1 mM nicotinate concentrations. Long-chain nicotinates, which could not be administered in medium due to their poor aqueous solubility, increased NAD/NADP levels if administered in PFOB at concentrations ≥10 mM. These findings suggest that even highly lipophilic prodrugs can partition out of the PFOB phase into cells, where nicotinic acid is released and converted to NAD. Thus, PFOB may be a novel and biocompatible vehicle for the delivery of lipophilic prodrugs of nicotinic acid and other drugs directly to the lung of laboratory animals and humans.
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## 1. Introduction

Perfluorocarbon liquids have great potential as materials for a variety of biomedical applications. The most prominent fluorocarbon liquid, perfluorooctyl bromide (PFOB; perflubron; $C_8F_{17}Br$), has been extensively studied as a contrast agent and a blood substitute in both preclinical and clinical studies (Riess, 2005). Over the past decade, research has also focused on the use of PFOB for liquid ventilation, a novel supportive ventilatory technique (Wolfson and Shaffer, 2005). During liquid ventilation PFOB is employed as a vehicle for the transport of oxygen into and carbon dioxide out of the lung. This treatment modality utilizes the high solubility of oxygen and carbon dioxide in PFOB; the capacity of PFOB to carry oxygen is up to three times greater than that of blood (Lehmler et al., 1999). PFOB is lipophobic and hydrophobic, stable at room temperature, exhibits a higher mass density than both water and soft tissue, and has a low surface tension and viscosity as well as a high vapor pressure (Lehmler et al., 1999), properties that make PFOB highly suitable for the aforementioned biomedical applications.

In addition to being applied as a vehicle for oxygen during liquid ventilation, PFOB and other fluorocarbon liquids are envisioned to serve as drug delivery vehicles, especially to the lung (Lehmler, 2007; Lehmler et al., 1999; Riess and Krafft, 1997, 1998, 1999). The advantages of such a novel pulmonary drug delivery system compared to conventional approaches include direct delivery of the drug to the lung with high concentrations of the drug at the lung parenchyma (Waldrep et al., 1997), avoiding the first pass effect and systemic distribution, equal distribution of the drug within the entire lung (i.e. by replacing

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edematous fluid) (Hirschl et al., 1994; Lehmler, 2007; Lehmler et al., 1999), and controlled release of the drug which further reduces systemic toxicity (Keller, 1999). The perfluorocarbon-drug formulation can also be administered intratracheally to specific regions of the lung, which further reduces systemic toxicity by delivering the entire dose of the drug to the desired lung area.

One of the most significant obstacles to the clinical application of PFOB as a solvent for the administration of drugs to the diseased lung is the extremely poor solvent properties of PFOB (Lehmler, 2007; Lehmler et al., 1999). Although an excellent solvent for oxygen and carbon dioxide, perfluorocarbon liquids are notoriously poor solvents for typical drug molecules (Patrick, 1971). Several efforts to deliver biologically active compounds, drugs or anesthetics, with PFOB to the lung have been reported (Cullen et al., 1999; Fox et al., 1997; Franz et al., 2001; Kimless-Garber et al., 1997; Zelinka et al., 1997). Most of these studies employed dispersions and not solutions of the drug of interest in PFOB. Disadvantages of these approaches include problems with stability of the formulation, particle size, dose reliability, dispersion consistency and ultimately bioavailability as well as ventilation abnormalities and impairments in respiratory gas exchange and pulmonary mechanics. A variety of methods, including solubilizing agents (Williams et al., 1998) as well as liquid (Butz et al., 2002; Courrier et al., 2003; Courrier et al., 2004a,b; Sadtler et al., 1996) and solid (Cullen et al., 1999) emulsions have, therefore, been studied to increase the solubility of drugs in PFOB.

Another approach to improve the solubility of a PFOB-insoluble drug in PFOB is to use a PFOB-soluble prodrug (Hsu et al., 2003; Lehmler et al., 1999). Such a prodrug is obtained by chemical modification of a functional group of the parent drug with a fluorophilic promoiety, for example by esterification. The sole function of this promoiety is to enhance the solubility of the drug in PFOB. The parent drug is released *in vivo* by biological or chemical degradation of the prodrug. We have shown that nicotinates are promising prodrugs of nicotinic acid (Hsu et al., 2003), a precursor of $NAD^+$ (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate) that is under investigation for the prevention of skin carcinogenesis (Jacobson et al., 1999) and has been proven beneficial against bleomycin- and cyclophosphamide-induced lung injury (Gurujeyalakshmi et al., 2000a,b; Venkatesan and Chandrakasan, 1994). Specifically, nicotinates are soluble in PFOB and, depending on their chemical structure, release the parent drug nicotinic acid by both chemical and enzymatic hydrolysis with half-life's ranging from a few minutes to several weeks (Hsu et al., 2003).

We herein investigate if the proposed prodrug approach is indeed suitable to administer nicotinates using PFOB as a vehicle to cells in culture. In a first step, the cytotoxic effect of a series of nicotinates dissolved in medium or in PFOB was assessed. Subsequently the increase of cellular NAD(H) and NADP(H) levels following exposure the nicotinate-PFOB solutions was determined. The results from these cell culture studies are discussed in the context of the partition-coefficients of the nicotinates between lipophilic, aqueous and fluorous environments.

## 2. Materials and methods

### 2.1. Synthesis and characterization of fluorinated nicotinate esters

The structures of the nicotinates used in this study are shown in Fig. 1. The four hydrocarbon nicotinates were obtained from Acros Organics (NJ, USA) and purified by Kugelrohr distillation prior to use. Fluorinated nicotinic acid esters were synthesized as described previously (Hsu et al., 2003; Lehmler et al., 2005). In short, nicotinic acid (15 mmol; Acros Organics, NJ, USA) was mixed with the alcohol (10 mmol) in anhydrous dichloromethane ($CH_2Cl_2$). Dicyclohexylcarbodiimide or dicyclopropyl carbodiimide (11 mmol) and dimethyl amino pyridine (1.2 mmol) were added to the solution. The mixture was stirred at room temperature for 16–22 h, the white precipitate was filtered off and the solvent was removed under vacuum. The product was purified by Kugelrohr distillation or, in the case of longer chain nicotinates, by column chromatography (Silica 60–200 mesh, hexane:ethyl acetate 9:1). Typical yields were greater than 80%. The purity of the esters was determined by gas chromatography to be >98%. The spectroscopic data of all compounds were in agreement with previously reported data (Hsu et al., 2003; Lehmler et al., 2005).

#### 2.1.1. 2,2,2-Trifluoroethyl nicotinate C2F3

$^1$H (400 MHz, [d6] acetone) $\delta$: 4.98 ($OCH_2CF_2H$, 2H, q, $J = 8.8$ Hz), 7.57 ($OCH_2CF_2H$, 1H, dd, $J = 8.2$ and 4.7 Hz), 8.35 (CH, 1H, dt, $J = 8.0$ and 1.7 Hz), 8.83 (CH, 1H, dd, $J = 4.7$ and 1.4 Hz), 9.16 (CH, 1H, d, $J = 2.2$ Hz); $^{13}$C (100 MHz, [d6] acetone) $\delta$: 60.95 (q, $J = 36.1$ Hz), 124.57 (Q, $J = 274.9$ Hz), 124.76, 125.63, 138.01, 151.59, 155.35, 164.54; $^{19}$F (282 MHz, [d] chloroform) $\delta$: −74.1 ($CF_3$); GC/MS, *m/z* (relative intensity, %): 205 ($C_8H_6F_3NO_2^{\bullet+}$, 28), 177 (15), 106 (100), 78 (93), 51 (53).

#### 2.1.2. 2,2,3,3,3-Pentafluoropropyl nicotinate C3F5

$^1$H (400 MHz, [d6] acetone) $\delta$: 4.82 ($OCH_2CF_3$, 2H, t, $J = 12.6$ Hz), 7.45 (CH, 1H, dd, $J = 8.2$ and 4.8 Hz), 8.32 (CH, H, dt, $J = 8.0$ and 1.8 Hz), 8.84 (CH, 1H, d, $J = 4.8$ Hz), 9.26 (CH, 1H, d, $J = 2.1$ Hz); $^{13}$C (100 MHz, [d6] acetone) $\delta$: 59.57 (t, $J = 28.7$ Hz), 123.28, 124.17, 137.06, 150.88, 154.08, 163.36; $^{19}$F (282 MHz, [d] chloroform) $\delta$: −84.16, −123.7; GC/MS, *m/z* (relative intensity, %): 255 ($C_9H_7F_4NO_2^{\bullet+}$, 100), 227(51), 106 (100), 78 (100), 51 (66).

#### 2.1.3. 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-Pentadecafluorooctyl nicotinate C8F15

$^1$H (400 MHz, [d6] acetone) $\delta$: 5.11 ($OCH_2CF_2$, 2H, t, $J = 13.8$), 7.58 (CH, 1H, dd, $J = 8.2$ and 4.8 Hz), 8.35 (CH, 1H, dt, $J = 8.0$ & 2.7 Hz), 8.85 (CH, 1H, dd, $J = 4.8$ and 1.7 Hz), 9.16 (CH, 1H, d, $J \approx 1.5$ Hz); $^{13}$C (100 MHz, [d6] acetone) $\delta$: 60.95 (t, $J = 27.4$ Hz), 124.85, 125.60, 138.05, 151.66, 155.50, 164.58; $^{19}$F (282 MHz, [d] chloroform) $\delta$: −81.4 ($CF_3$,), −119.8, −119.9, −122.5, −123.3, −123.4, −126.7; GC/MS, *m/z* (relative intensity, %): 505 ($C_{14}H_6F_{15}NO_2^{\bullet+}$, 33), 486 (9), 106 (100), 78 (58).

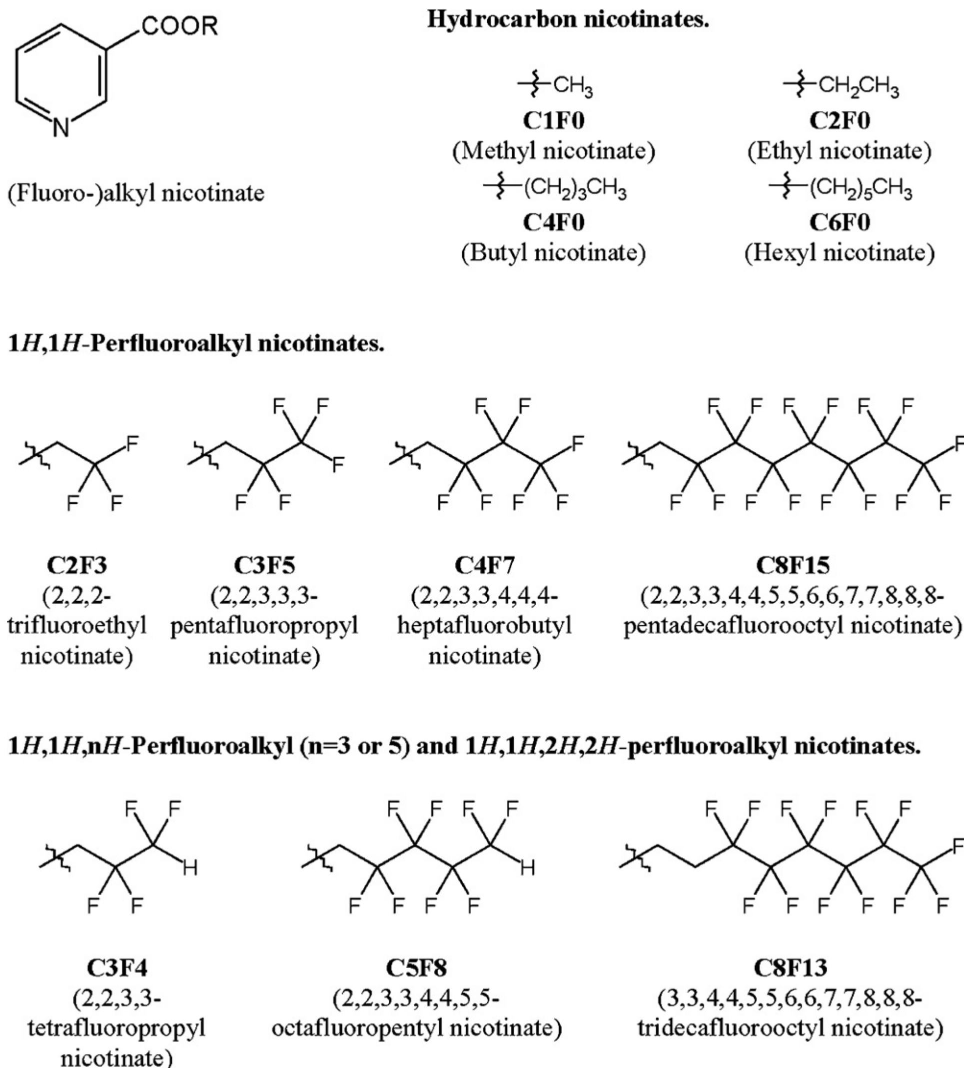


Fig. 1. Structures and IUPAC names (in parentheses) of partially fluorinated nicotinates. The abbreviations in bold indicate the length of the alcohol moiety and the number of fluorine atoms.

*2.1.4. 2,2,3,3-Tetrafluoropropyl nicotinate C3F4*

$^{1}$H (400 MHz, [d] chloroform) $\delta$: 4.75 ($OCH_2CF_2CHF_2$, 2H, t, $J = 12.8$), 6.01 ($CH_2CF_2CHF_2$, tt, $J = 53.0$ and 3.3 Hz), 7.42 (CH, 1H, dd, $J = 8.0$ and 4.8 Hz), 8.30 (CH, 1H, dt, $J = 8.0$ and 1.8 Hz), 8.82 (CH, 1H, dd, $J = 4.0$ and 1.6 Hz), 9.23 (CH, 1H, d, $J = 2.0$ Hz); $^{13}$C (100 MHz, [d] chloroform) $\delta$: 60.03 (t, $J = 29.1$ Hz), 109.4 (Tt, $J = 250.4$ and 37.6 Hz), 114.0 (Tt, $J = 249.8$ & 28.7 Hz), 123.39, 124.55, 137.21, 151.05, 154.15, 163.81; $^{19}$F (282 MHz, [d] chloroform) $\delta$: −123.3 ($CF_3$), −137.2 ($CF_2$); GC/MS, *m/z* (relative intensity, %): 237 ($C_9H_7F_4NO_2^{\bullet+}$, 24), 106 (100), 78 (64), 51 (49).

*2.1.5. 2,2,3,3,4,4,5,5-Octafluoropentyl nicotinate C5F8*

$^{1}$H (400 MHz, [d6] acetone) $\delta$: 5.06 ($OCH_2CF_2$, 2H, t, $J = 14.0$ Hz), 6.77 ($OCH_2(CF_2)_3$ $CHF_2$, tt, $J = 51.0$ and 5.5 Hz), 7.58 (CH, 1H, dd, $J = 8.0$ and 4.4 Hz), 8.35 (CH, 1H, dt, $J = 8.0$ and 1.8 Hz), 8.85 (CH, 1H, dd, $J = 4.4$ and 1.5 Hz), 9.17 (CH, 1H, d, $J \approx 1.5$ Hz); $^{13}$C (100 MHz, [d6] acetone) $\delta$: 60.93 (t, $J = 29.2$ Hz), 124.81, 125.63, 138.03, 151.63, 155.42, 164.61; $^{19}$F (282 MHz, [d] chloroform) $\delta$: −119.6, −125.5, −130.8, −137.4; GC/MS, *m/z* (relative intensity, %): 337 ($C_{11}H_7F_8NO_2^{\bullet+}$, 21), 106 (100), 78 (70), 51 (29).

*2.2. Determination of partition coefficients*

Estimates of the octanol–water partition coefficient were obtained using the software program from Advanced Chemistry Development Inc. (Ontario, Canada). The partition coefficients for the hydrocarbon and fluorocarbon prodrugs in a perfluoro(methylcyclohexane)–toluene system or PFOB–water were determined by FID gas chromatography (Varian CP-3800) analysis of the fluorocarbon phase (Rocaboy et al., 2000). The nicotinic acid esters were initially dissolved in 3 mL or 5.5 mL volumes of fluorinated solvent (PFOB or PFMCH) to achieve a known concentration in the range of 1–7.0 mM. Volume ratios of 1:1 and 1:5 in PFMC–toluene systems and 1:1, 1:5, 1:10, or 1:30 PFOB–water were used to achieve measurable equilibrium concentration differences in the fluorocarbon phase. The stir flask method was employed (Danielsson and Zhang, 1996; Hsu et al., 2003), in which equilibration was facilitated by stirring only the

denser (fluorocarbon) phase. All experiments were performed in temperature controlled environments at 25 °C.

Duplicate initial and equilibrium samples of the fluorocarbon phase were injected onto a 95% dimethylpolysiloxane stationary phase capillary column (Varian fused silica column/CP-SIL8 CB, 15 m × 0.25 mm (ID) × 0.25 μm). All of these 0.5 mL samples were spiked with 5 μL of internal standard (naphthalene-d8) in methylene chloride. Relative ratios of the nicotinic acid ester peak to the internal standard peak were used to determine the concentration of test compound in the fluorocarbon phase at equilibrium. These concentrations were measured from 4-point calibration curves. The corresponding concentration in the water or toluene phase was determined by material balance based on the depletion of the fluorinated phase. The log of the partition coefficient, log $K_p$, was then calculated using the equations below:

$$\log\ K_p\left(\frac{\text{PFMC}}{\text{toluene}}\right) = \log\left(\frac{[\text{Solute}]_{\text{PFMC}}}{[\text{Solute}]_{\text{toluene}}}\right) \quad (1)$$

or

$$\log\ K_p\left(\frac{\text{PFOB}}{\text{water}}\right) = \log\left(\frac{[\text{Solute}]_{\text{PFOB}}}{[\text{Solute}]_{\text{water}}}\right) \quad (2)$$

### 2.3. *Purity of perfluorooctyl bromide (PFOB)*

Two batches of PFOB were obtained from Sigma–Aldrich (Milwaukee, WI, USA) and used without further purification. The purity of each PFOB batch was determined using $^{19}$F NMR spectroscopy (Vyas et al., 2007). Known amounts of the PFOB and 1,4-bis(trifluromethyl)benzene (Aldrich, Milwaukee, WI, USA) as internal standard were dissolved in [d6] acetone and the $^{19}$F NMR spectra were recorded at 25 °C on a Bruker Avance 300 MHz NMR instrument equipped with a 5-mm QNP {$^{1}$H, $^{13}$C, $^{19}$F, and $^{31}$P} probe. Data were acquired with TD = 256 K data points and processed at size SI = 256 K. The acquisition time ($A$) and recycling delay time ($D_1$) were equal to approximately 10.00 s. The integral heights of the internal standard and the respective PFOB signal were used to calculate the purity of each PFOB batch.

### 2.4. *Cell culture experiments*

#### 2.4.1. *Cell line and cell maintenance*

All cell culture experiments were performed using the NCI-H358 cell line (American Tissue Collection, Rockville, MD, USA), a human non-small cell lung cancer cell line with an average carboxylesterase activity ($1.19 \times 10^{-4}$ mol pNP/min/g prot). NCI-H358 cells were cultured in RPMI medium supplemented with 10% FBS (fetal bovine serum), 2 mM sodium pyruvate, penicillin (50 IU mL$^{-1}$), streptomycin (50 μg mL$^{-1}$), and 10 mM HEPES. All products were obtained from Gibco BRL (Grand Island, NY, USA). Cultures were maintained in 75 cm$^2$ culture flasks (Costar, Cambridge, MA, USA) in a humidified incubator at 37 °C and 5% $CO_2$.

#### 2.4.2. *Cell viability evaluation of nicotinates in medium and PFOB with human lung NCI-H358 cells using the resazurin reduction assay*

NCI-H358 cells were seeded into 96-well plates (Costar, Cambridge, MA, USA) and cultured for several days to reach confluence. At this point the medium was replaced with 30 μL of neat PFOB or fresh medium (control) or 30 μL of a nicotinate solution in PFOB or medium and covered with a silicon membrane to reduce volatilization. After 3 h incubation the PFOB or medium with/without the respective nicotinate was carefully removed. The cells were washed twice with phosphate buffered saline (PBS, Gibco, Grand Island, NY, USA) and fresh culture medium was added. Effects on cell viability and growth were assessed at different time points using the resazurin reduction assay, which measures cell metabolic activity.

For the Resazurin assay the medium was replaced with fresh culture medium to which 20 μL of a 50 μM resazurin solution in HBSS (Gibco, Grand Island, NY, USA) had been added. After 2 h incubation the fluorescence intensity in each well was measured using a Tecan Genios Pro microplate reader (Tecan, Durham, NC, USA) and 535 nm excitation and 590 nm emission wavelengths. Living cells reduce the dark blue resazurin to the red resorufin. Cell survival was calculated by comparing the fluorescence intensity of the treated wells to the solvent controls and the effective concentrations that reduced the viable cells per well by 20% (EC20 values), were obtained from the dose–response curves. The EC20 instead of the more common EC50 was chosen, since our goal is to use these compounds at concentrations where they have no or hardly any negative effect on cell viability. For all experiments the EC20 values determined 3 days after the exposure ($\text{EC20}_{\text{day 3}}$) are presented, because our time course experiments showed that the toxic effect of these compounds is not acute but delayed. Furthermore, this time point appeared to be the most sensitive to see a negative effect on cell viability of the nicotinates in PFOB or medium as for some compounds a recovery was noted beginning at day 4.

#### 2.4.3. *Determination of cellular NAD/NADH and NADP/NADPH levels after exposure to nicotinates*

NCI-H358 cells were seeded in 96-well plates and allowed to reach confluency. The cells were exposed for 3 h to 30 μL of prodrug solutions in PFOB or in RPMI 1640 medium without niacin (Altanta Biologicals, Norcross, GA, USA) supplemented with 2% dialyzed FBS (Giboco BRL, Grand Island, NY, USA). The respective vehicle (i.e., PFOB or medium) was used as control. As in all previous experiments, the wells were coated with a silicon membrane to reduce volatilization of the PFOB and/or the respective nicotinate. The cells were washed twice with PBS and cultured in niacin (i.e. nicotineamide)-depleted RPMI medium for another 3 h. Cells were washed again twice with PBS and lysed by freezing at −80 °C overnight with 200 μL extraction buffer (100 mM Tris–HCl, 0.05% (v/v) Triton-X-100 and 10 mM EDTA at pH 8.0). NAD(H) and NADP(H) concentrations in the lysates were measured with an enzymatic cycling assay adapted for microplate readers (Jacobson and Jacobson, 1997).

Briefly, 40 μL lysate from each well was transferred to a 96-well plate and mixed with 10 μL of 20 mM MPS (Sigma, St. Louis, MO) and 50 μL premix solution for NAD(H) (1 mM MTT, 1 M ethanol, 10 mM EDTA and 100 mM Tris–HCl) or NADP(H) (1 mM MTT, 20 mM D-glucose-6-phosphate monosodium salt, 150 mM $MgCl_2$ and 100 mM phosphate buffer) determination, respectively. After 10 min at 37 °C, 10 μL of a solution containing 2 IU of glucose-6-phosphate dehydrogenase and 1 mg/mL alcohol dehydrogenase for NAD and NADP measurement, respectively, were added. The absorbance increase over time at 595 nm was monitored immediately using the Tecan Genios Pro microplate reader. The NAD(H) and NADP(H) concentrations were determined by comparing the rate of absorbance increase with those of appropriate NADH and NADPH (both from Sigma, St. Louis, MO, USA) standard solutions. The protein amount of the lysates was measured with the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) and used to calculate NAD(H) and NADP(H) levels per mg protein.

### *2.5. Statistical analysis of the cellular NAD/NADH and NADP/NADHP data*

Every exposure was done in triplicate or more and all experiments were performed at least twice in independent experiments. A linear mixed model was used to analyze for statistical significance. The comparison of the treated groups to the control was tested with the Dunnett's test. All statistical analyses were performed with the program SAS 9.1 (SAS Institute Inc, 2003). (*) $p < 0.05$ and (**) $p < 0.01$ was considered to be significant.

## 3. Results and discussion

### *3.1. Partitioning behavior of nicotinate prodrugs*

We determined or estimated the PFOB–water, PFMC–toluene and octanol–water partition coefficients to assess the ability of a series of nicotinate prodrugs (Fig. 1) to partition out of the PFOB phase and into the cell membrane. The tendency of a given nicotinate prodrug to partition out of the PFOB phase into water can be assessed using the PFOB–water partition coefficient (Lehmler et al., 1999). All nicotinates with <7 fluorine atoms had log partition coefficient values ranging from −0.78 to 1.36 and, with exception of C1F0 and C2F0, had a moderate preference for the PFOB phase (Table 1). The slightly negative partition coefficients of the two short-chain hydrocarbon nicotinates C1F0 and C2F0 were not surprising as these compounds are relatively polar and highly water-soluble. We were unable to determine the PFOB–water partition coefficient of the four long-chain, fluorinated nicotinates because these nicotinates, like their hydrocarbon analogues (Lehmler and Bummer, 2005; Lehmler et al., 2005), adopt surfactant-like properties with increasing chain length. These surface active properties hindered the separation of the PFOB and the water phase (due to emulsion formation) and resulted in solute concentration-dependent partition behavior (due to the potential self-assembly of the surfactants). Based on the detection limits of our analytical assay we estimate that the PFOB–water partition coefficients of these compounds were $\log K_p$ (PFOB/$H_2O$) >2.2, i.e. these highly fluorinated nicotinates (≥7 fluorine atoms) had a significant affinity for the PFOB phase. Overall, the PFOB–water partition coefficient did not appear to be a general tool to assess the ability to deliver an amphiphilic prodrug from a fluorocarbon phase because of the challenges associated with both the direct measurement of the prodrug in the water phase and the measurement of the fluorocarbon phase prodrug concentration in the presence of water.

Alternatively, the fluorophilicity of the nicotinates can be described using the PFMC–toluene partition coefficient. This partition coefficient has the advantage that it can be easily determined experimentally or estimated using a variety of theoretical approaches (Daniels et al., 2004; Duchowicz et al., 2004; Kiss et al., 2001). The PFMC–toluene partition coefficients of all nicotinates fell into a relatively narrow range from $\log K_p$ (PFMC/toluene) = −2.62 to −0.70 (Table 1). The only exception was C8F15 which was marginally fluorophilic with $\log K_p$ (PFMC/toluene) = 0.13. The PFMC–toluene partition coefficient decreased with increasing chain length in the hydrocarbon nicotinate series, whereas the opposite trend was true for the fluorocarbon series. These observations are in agreement with established empiric rules to predict the fluorophilicity

Table 1
PFOB–water, PFMC–toluene and octanol–water partition coefficients of hydrocarbon and fluorocarbon nicotinates at 25 °C[a]

| Nicotinate | | $\log K_p$ (PFOB/$H_2O$) | $\log K_p$ (PFMC/$H_3CC_6H_5$) | $\log K_p$ ($C_8H_{17}OH/H_2O$) |
|---|---|---|---|---|
| Hydrocarbon nicotinates | C1F0 | −0.78 ± 0.04 | −1.72 ± 0.21 | 0.9 |
| | C2F0 | −0.157 ± 0.01 | −2.22 ± 0.41 | 1.4 |
| | C4F0 | 0.96 ± 0.01 | −2.15 ± 0.02 | 2.5 |
| | C6F0 | 1.23 ± 0.18 | −2.62 ± 0.02 | 3.5 |
| 1*H*,1*H*-Perfluoroalkyl nicotinates | C2F3 | 0.48 ± 0.03 | −1.64 ± 0.12 | 2.2 |
| | C3F5 | 0.75 ± 0.09 | −1.50 ± 0.08 | 2.7 |
| | C4F7 | >2.2 | −1.03 ± 0.01 | 4.2 |
| | C8F15 | >2.2 | 0.13 ± 0.01 | 10.2 |
| 1*H*,1*H*,*nH*-Perfluoroalkyl nicotinates: ($n$ = 3 or 5) | C3F4 | 0.55 ± 0.01 | −1.63 ± 0.03 | 2.3 |
| | C5F8 | >2.2 | −1.62 ± 0.11 | 4.1 |
| 1*H*,1*H*,2*H*,2*H*-Perfluoroalkyl nicotinate | C8F13 | >2.2 | −0.70 ± 0.16 | 6.2 |

[a] Values in the first two columns are means of two independent experiments with variations from the mean (±one standard deviation) given in parentheses.

of a molecule. Typical fluorophilic molecules have a fluorine content >60 wt.%, at least one long fluorous tail, and contain no or few functional groups allowing attractive intermolecular interactions (Kiss et al., 2001). All nicotinates investigated, including C8F15, had a fluorine content below 60 wt.% and, with exception of C8F13 and C8F15, the fluorous tail was very short. Therefore, the nicotinates shown in Fig. 1 are not expected to be fluorophilic.

The octanol–water partition coefficient is a measure of the lipophilicity of the nicotinates and, in the absence of active transport mechanism, directly correlated with the diffusion of compounds through the cell membrane into the cell. As shown in Table 1, the octanol–water partition coefficient of the nicotinates used in this study covered over five orders of magnitude and increased with increasing chain length and increasing degree of fluorination. Similarly, the PFOB–water and PFMC–toluene partition coefficients covered at least two orders of magnitude. Thus, the eleven nicotinates investigated in this study cover a broad range of physicochemical properties, which makes this series of prodrugs ideally suited for the cell culture studies described below.

### 3.2. Purity and cytotoxic effect of neat PFOB

Many perfluorocarbon compounds are synthesized by an electrochemical fluorination process (Lehmler, 2005). Perfluorocarbon compounds synthesized by this route are typically highly impure and, besides containing various organofluorine compounds, can contain trace amounts of toxic inorganic impurities such as heavy metals. We determined the purity of PFOB using $^{19}$F NMR spectroscopy to verify that PFOB obtained from commercial sources was not synthesized by this route and did not contain potentially toxic impurities. The two PFOB batches used in this study were 95.7 ± 0.5% and 96.9 ± 1.0% PFOB, respectively, and only the fluorine signals of PFOB were observed. Because of the absence of other organofluorine compounds, the two PFOB batches were considered to be suitable for cell culture studies and no attempts were made to further purify the PFOB.

In the next step, cell culture studies in NCI-H358 cells were performed to verify that PFOB was non-toxic. During liquid ventilation and the proposed pulmonary administration of nicotinates dissolved in PFOB the epithelial cells of the lung would be covered with a thin film of PFOB. To simulate the *in vivo* conditions we created a PFOB–liquid interface by overlying confluent NCI-H358 cells with 30 μL of neat PFOB, an amount that was sufficient to fully cover the cells over exposure periods up to 7 h. This approach is similar to recent cell culture studies analyzing the protective effect of PFOB against oxidative injury (Babu et al., 2005; Rotta et al., 2003). Although the cells were kept during this time in the absence of nutritive medium, our studies showed that overlaying the cells with PFOB did not result in any immediate or long-term cytotoxicity for short-term exposure times up to 7 h (Fig. 2). These findings are in agreement with cell culture studies employing pharmaceutical grade PFOB formulation (Babu et al., 2005; Rotta et al., 2003) or other perfluorocarbon liquids (Lowe et al., 1998).

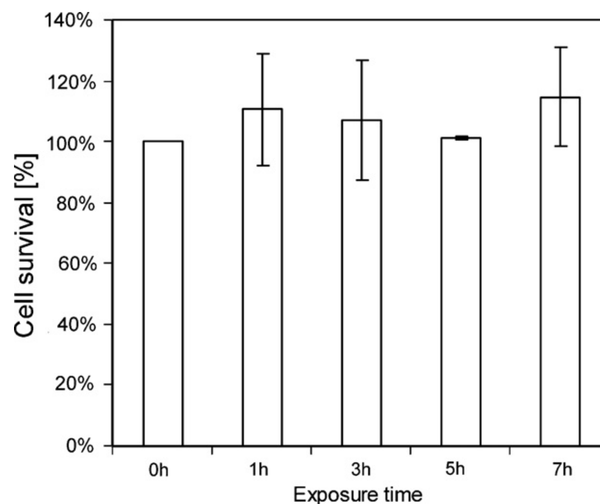


Fig. 2. Cytotoxicity of 1–7 h exposure to neat PFOB in confluent NCI-H358 cells. The data are given as percent of medium controls and represent the averages of three independent experiments ± standard deviation.

### 3.3. Cytotoxic effect of PFOB–nicotinate solutions compared to nicotinates in medium

We investigated the cytotoxic effect of nicotinate–PFOB solutions by exposing confluent NCI-H358 cells to nicotinates dissolved either in PFOB or medium. The goal of these studies was to determine the EC20 (i.e., the lowest observable adverse effect level) of the PFOB–nicotinate solutions for the subsequent determination of cellular NAD(H) and NADP(H) levels. An exposure time of 3 h was chosen because drug transport studies in confluent cells are typically performed in this time period and our first exploratory tests indicated that the nicotinates significantly increased cellular NAD(H) levels in this time (see our results below). In our cell culture experiments the PFOB was removed after the 3 h exposure time and the cells were maintained with cell culture medium for up to 6 days with daily measurements of resazurin reduction to assess potential adverse effects of the nicotinate and/or PFOB. Under these conditions, the solutions of all nicotinate prodrugs in both medium and PFOB displayed moderate-to-low toxicities with EC20's in the millimolar range (Table 2). These initial toxicity results suggest that potentially large amounts of these prodrugs can be safely administered *in vivo*.

The results from a time course experiment with 25 mM solutions of C2F3, a fluorinated nicotinate, in medium and PFOB are shown in Fig. 3. At this concentration C2F3 delivered in cell culture medium did not have a strong effect on the short and long-term survival of the cells over the time period tested. At the same concentration, PFOB-dissolved C2F3 strongly reduced the cell survival beginning at day 2. In other words, the same concentration that caused only a minor effect when delivered in medium resulted in an 80% reduction in living cells if delivered in PFOB. As shown in Table 2, the cytotoxicity of the other nicotinates also depended strongly on the delivery medium. For example, C2F3 and other short chain nicotinates had a lower $EC20_{day\ 3}$ if delivered in PFOB, whereas nicotinates with a longer chain, for

Table 2
Cytotoxic effect of nicotinates towards confluent human lung NCI-H358 cells measured 3 days after a 3 h exposure to the nicotinate in PFOB or cell culture medium (averages ± S.D. of three or (*) mean of two independent experiments)

| Nicotinate | | $EC20_{day\ 3}$ in PFOB (mM) | $EC20_{day\ 3}$ in medium (mM) |
|---|---|---|---|
| Hydrocarbon nicotinates | C1F0 | 11.3 ± 3.2 | >50 |
| | C2F0 | 13.7 ± 4.5 | >40 |
| | C4F0 | 11.3 ± 3.2 | 8.5 ± 0.7 |
| | C6F0 | 9.8 ± 2.5 | >2 |
| 1*H*,1*H*-Perfluoroalkyl nicotinates | C2F3 | 8.7 ± 3.9 | >25 |
| | C3F5 | 2.9* ± 0.5 | >15 |
| | C4F7 | 28.5 ± 17.7 | >7.5 |
| | C8F15 | >500 | n.d. |
| 1*H*,1*H*,*nH*-Perfluoroalkyl nicotinates ($n = 3$ or 5) | C3F4 | 3.8 ± 1.8 | >20 |
| | C5F8 | 5.9 ± 0.1 | n.d. |
| 1*H*,1*H*,2*H*,2*H*-Perfluoroalkyl nicotinate | C8F13 | 84.0* ± 2.0 | n.d. |

n.d. = not determined due to limited solubility in cell culture medium.

example C4F0, appeared to have a similar toxicity in either delivery vehicle. The low solubility of these nicotinates in medium, however, prevented a closer investigation of this observation.

### *3.4. Effect of nicotinate–PFOB and nicotinate-cell culture medium solutions on cellular NAD/NADH and NADP/NADHP levels in lung NCI-H358 cells*

We and others have shown that nicotinates release nicotinic acid by chemical and/or enzymatic hydrolysis (Hsu et al., 2003; Wernly-Chung et al., 1990). Therefore, nicotinates are potential prodrugs for nicotinic acid, an important precursor of $NAD^+$ and $NADP^+$ (a secondary product in the NAD biosynthesis pathway, i.e. $NAD^+ + ATP \rightarrow NADP^+ + ADP$). In fact, nicotinates have been shown to increase NAD levels in cells in culture and *in vivo*, thus making them potential chemopreventive agents (Gensler et al., 1999; Jacobson et al., 1995; Jacobson et al., 1999). Therefore the increase of cellular NAD and NADP levels was investigated

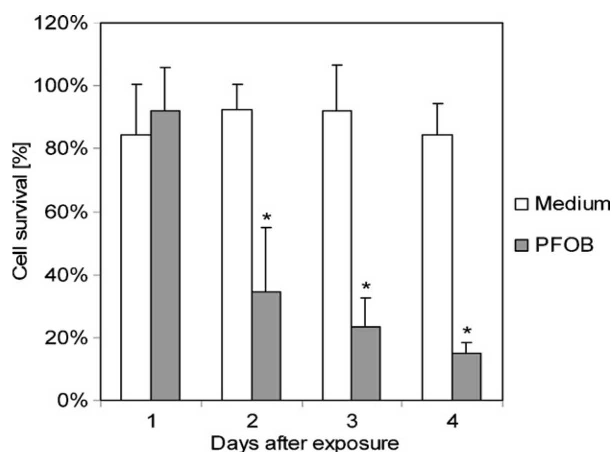


Fig. 3. Cytotoxic effect of a 25 mM solution of C2F3 in cell culture medium or PFOB. Confluent NCI-H358 cells were exposed for 3 h and cell survival was assessed at 1, 2, 3 and 4 days after exposure using the resazurin reduction assay. The data represent the averages of three independent experiments ± standard deviation (* = significantly different from day 1 at $p < 0.05$).

as an indicator of the successful delivery of nicotinates to the lung following the experimental design used for the determination of the $EC20_{day\ 3}$. Such a generalized method of assessment not only allows to screen an extensive series of nicotinates compared to the more difficult and limited direct measurement of the cellular levels of individual nicotinates, but also demonstrates the bioavailability of the nicotinic acid moiety from the nicotinate as precursor for cellular cofactor synthesis.

The normal level of NAD(H) and NADP(H) in our NCI-H358 cells was roughly around 6 and 2.3 μmol/g protein, respectively. Initial time-course experiments showed that levels of both (H) and NADP(H) displayed a statistically significant increase after a 3 h exposure to a nicotinate or nicotinic acid itself (data not shown). This exposure time was therefore used in all subsequent experiments. At a concentration of 1 mM, which is below the $EC20_{day\ 3}$ of all nicotinates (Table 2), all short-chain nicotinates significantly increased NAD(H) levels to a similar extent, by about 30–50% above control levels, when administered in PFOB or medium (Fig. 4). The exception was C2F0, which did not significantly increase NAD(H) levels when administered in cell culture medium. NADP(H) levels were also significantly elevated by all PFOB-delivered nicotinates, although to a slightly smaller extent, by about 20–40%, than the NAD(H) levels; however, the increase in intracellular NADP(H) levels after treatment with nicotinates in medium reached significance with only two compounds, C1F0 and C4F7 (Fig. 4).

The two long chain nicotinates C8F13 and C8F15 were not tested in medium because of their poor solubility in water (Table 2). However, because of their high degree of fluorination both nicotinates are highly soluble in PFOB, thus allowing us to administer these compounds at concentrations up to 500 mM (Fig. 5). C8F13 was only tested up to 50 mM because higher concentrations were cytotoxic. Neither nicotinate effected cellular NAD(H) and NADP(H) levels at a 1 mM concentration. However, a statistically significant increase in both NAD(H) and NADP(H) levels could be observed at higher concentrations, thus suggesting that these highly lipophobic and hydrophobic nicotinates, which are not soluble enough in water for drug deliv-

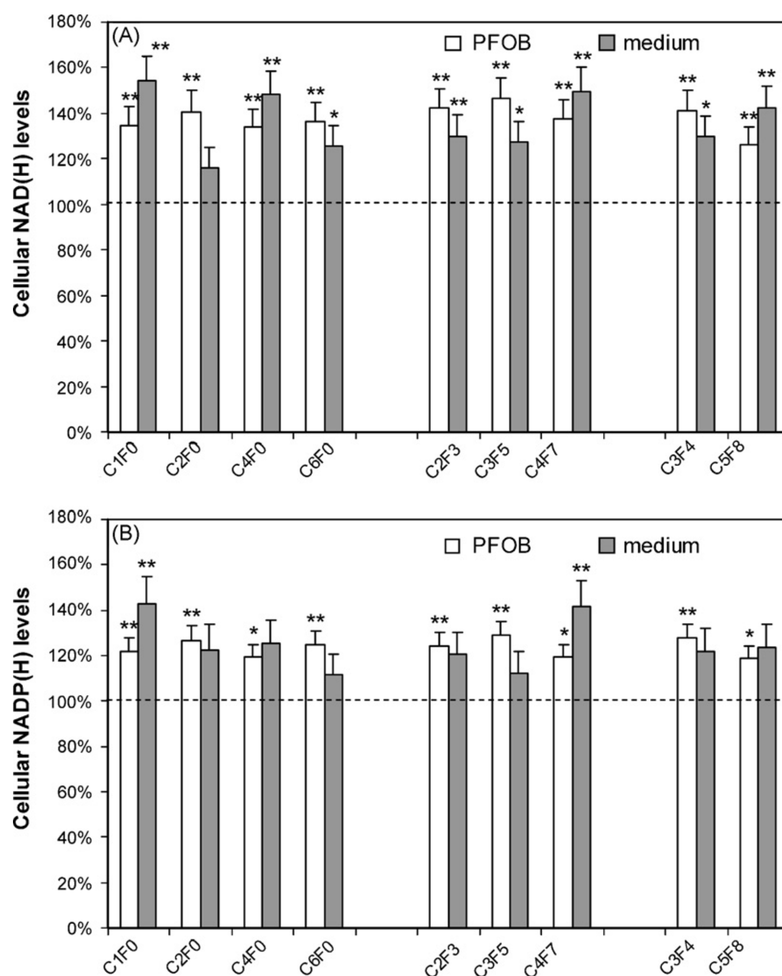


Fig. 4. Increase in cellular (A) NAD(H) and (B) NADP(H) levels measured 3 h after a 3 h exposure to a 1 mM solution of the respective nicotinate in PFOB or cell culture medium. The data represent the averages of three independent experiments ± standard error.

ery, can be successfully delivered to cells in culture using PFOB as vehicle.

### *3.5. Trends in the partitioning behavior of nicotinates to their biological effects*

Both hydrocarbon and fluorocarbon nicotinate prodrugs are expected to partition more readily into cells as their chain length and/or degree of fluorination increases. For example, we have recently shown that some fluorinated nicotinates have higher tendency to partition into model lipid bilayers compared to their hydrocarbon analogues (Lehmler and Bummer, 2005). To examine how the expected partitioning behavior into the bilayer affects the observed cytotoxicity of fluorinated nicotinates, trends in the $EC20_{day\ 3}$ were analyzed as a function of the octanol–water coefficient, a measure of lipophilicity, and the PFMC–toluene partition coefficient, a measure of fluorophilicity (Fig. 6). For this small series of $–CF_3$ terminated nicotinates, toxicity decreased with increasing lipophilicity or fluorophilicity (i.e., with an increase in fluorinated chain length). C8F15 also followed this trend but was not included in Fig. 6 because its $EC20_{day\ 3}$ was not determined. Similarly, higher concentrations of the two highly lipophilic octyl nicotinates in PFOB were needed to cause a similar increase in cellular NAD levels as with less lipophilic compounds (Fig. 5). These observations suggest that the biological effect of the fluorinated nicotinates was limited by their ability to partition out of the PFOB delivery phase or the cell bilayer.

In contrast, the cellular uptake of short-chain hydrocarbon nicotinates and the two nicotinate prodrugs with the highly polar $–CF_2H$ terminus appeared to be independent of the various partition coefficients, i.e. neither the $EC20_{day\ 3}$ nor the relative increase in NAD and NADP levels showed any correlation with the lipophilicity or fluorophilicity of the nicotinates. Thus, partitioning from the PFOB phase or the cell membrane into the cell did not appear to limit the biological effect of this group of prodrugs. Other factors, such as the 3 h exposure time or the mechanism of cellular uptake (e.g., endocytosis of the PFOB solution) may be the factors determining the cellular uptake and, thus, the biological activity of these nicotinate prodrugs. Although our cell culture experiments did not show a correlation between the partition coefficients and the biological effects of the short term nicotinates, our results suggest that these nicotinates can still be administered directly to the lung using PFOB.

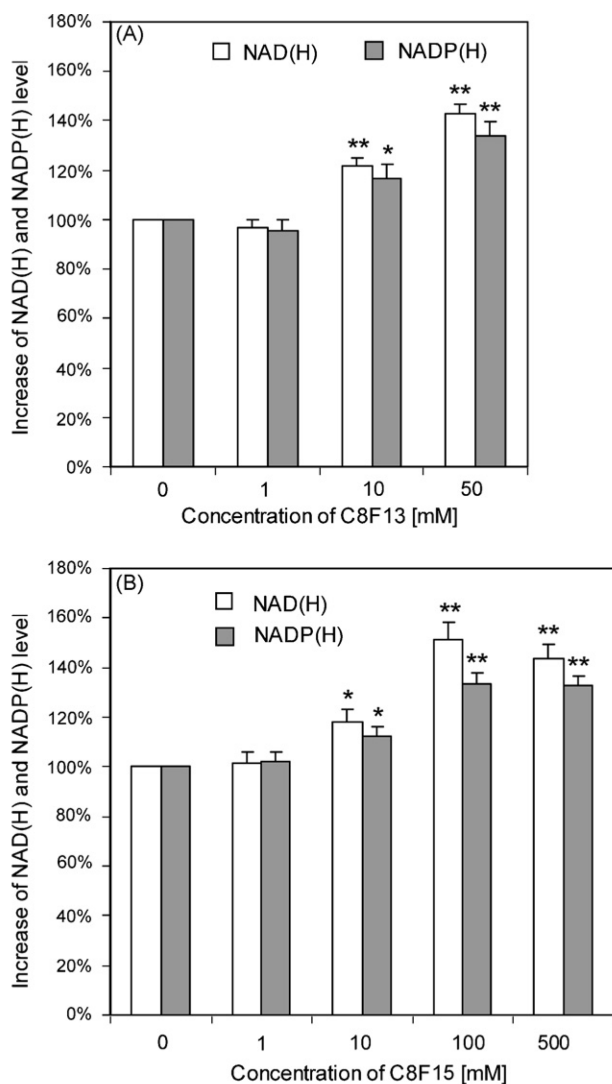


Fig. 5. Effect of solutions of the fluorinated octyl nicotinates (A) C8F13 and (B) C8F15 in PFOB on cellular NAD(H) and NADP(H) levels measured 3 h after a 3 h exposure. The data represent the averages of triplicates ± standard deviation.

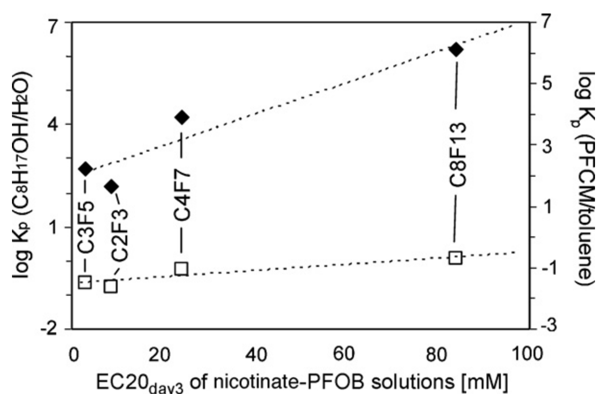


Fig. 6. Trends in the octanol–water (♦) or the PFMC–toluene partition coefficient (□) of fluorinated nicotinates and their $EC20_{day\ 3}$ after exposure to a nicotinate-PFOB solution in confluent NCI-H358 cells. The dashed lines represent linear fits of the data for the octanol–water ($R^2 = 0.91$) and the PFMC–toluene partition coefficient ($R^2 = 0.82$) as a function of $EC20_{day\ 3}$ values, and are intended to interpret general data trends.

## 4. Conclusions

The present study used a cell culture approach to evaluate the uptake of a series of hydrocarbon and fluorocarbon prodrugs of nicotinic acid using PFOB as drug delivery vehicle. Our results showed that PFOB can be used successfully to administer nicotinate prodrugs with a broad range of physicochemical properties to human lung cells in culture. For example, uptake of the nicotinate prodrugs from PFOB solutions was indirectly demonstrated by their measurable cytotoxicity, typically at millimolar concentrations. Interestingly, the toxicity of the nicotinates depended strongly on the vehicle used for their administration (culture medium or PFOB). More importantly, all nicotinates but one (C2F0 in medium) could increase cellular NAD levels at concentrations where they did not have a negative effect on cell viability if administered either with culture medium or PFOB. Typically only nicotinates delivered in PFOB also increased cellular NADP level. Even lipophilic and fluorophilic prodrugs, such as C8F15, partitioned out of the PFOB phase into cells and increased cellular NAD and NADP levels, although higher prodrug concentrations were needed. Due to their poor aqueous solubility these prodrugs could not be administered using medium as a vehicle. Interestingly, a correlation between the partition coefficients and the biological end points (i.e., $EC20_{day\ 3}$ and NAD/NADP levels) was only noted within the small series of fluorinated nicotinate prodrugs with a terminal $–CF_3$ group. In contrast, the toxicity and the ability to increase NAD and NADP levels of the short-chain nicotinate prodrugs was independent of their partition behavior, an observation that we currently cannot explain. Overall, the results from the present study suggest that PFOB is a useful vehicle to administer highly lipophilic prodrugs of nicotinic acid and other drug molecules directly to the lung *in vivo*.

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