We investigated estrogen-like properties of five perfluorinated compounds using a combination of three in vitro assays. By means of an E-screen assay, we detected the proliferation-promoting capacity of the fluorotelomer alcohols 1H,1H,2H,2H-perfluorooctan-1-ol (6:2 FTOH) and 1H,1H,2H,2H-perfluorodecan-1-ol (8:2 FTOH). The more widely environmentally distributed compounds perfluoro-1-octane sulfonate, perfluorooctanoic acid, and perfluorononanoic acid did not seem to possess this hormone-dependent proliferation capacity. We investigated cell cycle dynamics using flow cytometric analyses of the DNA content of the nuclei of MCF-7 breast cancer cells. Exposure to both fluorotelomer alcohols stimulated resting MCF-7 cells to reenter the synthesis phase (S-phase) of the cell cycle. After only 24 hr of treatment, we observed significant increases in the percentage of cells in the S-phase. In order to further investigate the resemblance of the newly detected xenoenestrogens to the reference compound 17β-estradiol (E2), gene expression of a number of estrogen-responsive genes was analyzed by real-time polymerase chain reaction. With E2, as well as 4-nonylphenol and the fluorotelomer alcohols, we observed up-regulation of trefoil factor 1, progesterone receptor, and PDZK1 and down-regulation of ERBB2 gene expression. We observed small but relevant up-regulation of the estrogen receptor as a consequence of exposures to 6:2 FTOH or 8:2 FTOH. The latter finding suggests an alternative mode of action of the fluorotelomer alcohols compared with that of E2. This study clearly underlines the need for future in vitro testing for specific endocrine-related endpoints. Key words: cell cycle, E-screen, fluorotelomer alcohols, real-time PCR, xenostrogen.

Vitamin K-dependent protein C (PC) and protein S (PS) are naturally occurring anticoagulants synthesized by the liver. The synthesis of these proteins is under translational control of vitamin K, a fat-soluble vitamin that is essential for the synthesis of many vitamin K-dependent proteins. The vitamin K cycle involves the oxidation of vitamin K by microsomal FAD-dependent glutathione reductase (GSR) and the reduction of the vitamin K-dependent proteins by vitamin K-dependent glutathione reductase (VKDR). The vitamin K cycle is dependent on the availability of vitamin K, which is synthesized by gut bacteria and integrated into dietary intake. The regulation of the vitamin K cycle is important for maintaining adequate levels of anticoagulant proteins and preventing vitamin K deficiency bleeding.

**Results**

Stimulation of MCF-7 cell proliferation. For this study, we adapted the E-screen assay to

Estrogen-like properties of fluorotelomer alcohols (FTOHs) from Gibco BRL Life Technologies (Paisley, Scotland). 1H,1H,2H,2H-Perfluorooctan-1-ol (6:2 FTOH) and 1H,1H,2H,2H-Perfluorodecan-1-ol (8:2 FTOH) were purchased from Interchim (Montlucon, France). We assessed the purity of the fluorotelomer alcohol standards by gas chromatography coupled to full-scan mass spectrometry in the electron impact (EI), negative chemical ionization (NCI), and positive chemical ionization (PCI) modes. No impurities could be detected in either EI or PCI mode. In NCI mode, several signals were observed. Retention times and full-scan mass spectra of these signals revealed that they were very closely related to the main signal. They were tentatively elucidated as branched isomers of the FTOHs.

**Cell culture.** MCF-7 human Caucasian breast adenocarcinoma cells (no. 86012803; European Collection of Cell Cultures, Salisbury, UK) were cultured in 25-cm² Nunc cell culture flasks (Nunc, Roskilde, Denmark) in standard growth medium (DMEM; Gibco BRL Life Technologies (Paisley, Scotland). 1H,1H,2H,2H-Perfluorooctanoic acid was purchased from Interchim (Montlucon, France). The growth medium was replaced by phenol red–free DMEM containing CSFBS. After incubation in estrogen-free medium for 72 hr, cells were exposed to E2 or test compounds at concentrations corresponding to the highest observed effect during the E-screen assay. After 24 hr, cells were harvested by trypsinization and washed twice in PBS. Next, cell nuclei were isolated and stained with propidium iodide (PI) for 1 hr as described by Vindelov et al. (1983). We performed flow cytometric analysis of cell cycle distribution and apoptosis with an LSRII flow cytometer with a 488-nm argon-ion laser (Becton Dickinson, San Jose, CA, USA). PI fluorescence was collected at bandpass 575/26 nm (FL2, red fluorescence channel) in the linear mode. For each measurement, data from 10,000 single cell events were collected, whereas cell aggregates and双倍 were gated out in the two-parameter histograms of pulse height to pulse width of PI fluorescence. We analyzed cell cycle histograms using ModFit LT 3.0 software (Variety Software House, Topsham, ME, USA).

**Gene expression analysis by reverse-transcription polymerase chain reaction (PCR).** MCF-7 cells were seeded and grown in estrogen-free medium in a manner analogous to that described above for flow cytometric analyses. After 48 hr exposure to E2 or test compounds, total RNA was extracted from the cell using the RNAeasy kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. RNA quantity and quality were evaluated using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, CA, USA). First-strand cDNA was synthesized using the Fermentas first-strand cDNA synthesis kit (MBI Fermentas Life Sciences, St. Leon-Rot, Germany). In brief, 1 µg total RNA was incubated with 0.5 µg oligo(dT)18 primer and annealed at 70°C for 5 min to denature RNA. Next, 20 U recombinant ribonuclease inhibitor and 1 mM dNTP mix were added to the RNA in the following reaction buffer: 50 mM Tris–HCl, pH 8.3; 50 mM KCl; 4 mM MgCl₂; and 10 mM dithiothreitol. cDNA synthesis was started by adding 40 U M-MulV (Moloney murine leukemia virus) reverse transcriptase at 37°C for 1 hr. Reaction was stopped by inactivation of the reverse transcriptase at 70°C for 10 min. The final volume (20 µL) was adjusted to 100 µL.

For the Lightcycler reaction, we prepared a master mix of the following components to the indicated end concentration: 9 µL water, 1 µL forward primer (0.5 µM), 1 µL reverse primer (0.5 µM), and 4 µL Lightcycler Fast Start DNA Master SYBR Green I reagent mixture (Roche). The Lightcycler glass capillaries were filled with 15 µL master mix, and 5 µL cDNA (50 ng reverse-transcribed total RNA) was added as the PCR template. We used the following experimental protocol: denaturation program (95°C for 5 min), amplification program (95°C for 5 sec, 58°C for 5 sec, 72°C for 13 sec, with a single fluorescence measurement at the end of DNA synthesis), melting curve program (55–95°C with a heating rate of 0.1°C/sec and a continuous fluorescence measurement), and finally a cooling step to 40°C. Expression changes of specific target genes were deduced from shifts of the crossing points for the target genes in exposed versus non-exposed cells and normalized by comparison with the internal control gene HPRT1. The “crossing point” is the point at which the fluorescence rises appreciably above the background fluorescence. The relative expression ratio of the gene under study normalized by the internal control HPRT1 gene was calculated using REST software (version 2; Pfaffl et al. 2002). We used the pairwise fixed reallocation randomization test included in the REST software to test the significance of the derived results. Each chemical treatment was performed in triplicate, which provided three replicate samples for the reverse-transcriptase (RT)-PCR analyses. To check amplification of the correct PCR products, we performed analysis by 1.5% agarose gel electrophoresis.

**Results**

Stimulation of MCF-7 cell proliferation. For this study, we adapted the E-screen assay to
growth-arrested MCF-7 cells are predominantly in the G0/G1-phase of the cell cycle, addition of (xeno-)estrogens makes cells proliferate again, shown by the marked increase in the percentage of cells in the S-phase after 24 hr of exposure.

In Figure 2, the histograms of DNA content show increases in S-phase as the result of exposures to the fluorotelomer alcohols and 4-NP. In Table 2, results are expressed as percentages of cells in the different phases of the cell cycle. The increases of cell numbers in the S-phase range from 6% (solvent control) to almost 35% (1 nM E2 and 4-NP), approximately 31% (50 µM, 6:2 FTOH), and approximately 29% (10 µM, 8:2 FTOH). PFOS, PFOA, or PFNA, at concentrations ≤ 50 µM, did not affect proliferation. As shown in Table 3, fluorotelomer alcohols stimulate MCF-7 cells at concentrations ranging between 10^{-6} and 10^{-3} M. At 10^{-2} M, the stimulatory effect is lost. These results corroborate the findings from the E-screen assay and demonstrate the reinduction of cell proliferation of growth-arrested MCF-7 cells within a much shorter exposure period.

Expression alterations of estrogen-responsive genes. Reverse-transcription PCR was performed to analyze the expression of specific estrogen-responsive biomarker genes after 48 hr of exposure. As presented in Figure 3, significantly high up-regulation of ESRI mRNA was observed with E2 (7.5×), 4-NP (3.8×), 6:2 FTOH (6.2×), and 8:2 FTOH (2.4×). A small up-regulation was also observed with PFOA (1.4×), and a small but significant down-regulation was observed upon exposure to PFOS (1.7×). Exposure to E2 induced very high PGR mRNA levels (30×), whereas significant up-regulations were seen with 4-NP (4.5×), 6:2 FTOH (10.4×), and 8:2 FTOH (2.4×). ESR1 was down-regulated upon exposures to E2 (1.33×) or 4-NP (2.1×). With 6:2 FTOH and 8:2 FTOH, however, small but significant up-regulations of ESR1 were observed (2.2× up with both compounds). PFOS exposure resulted in a significant small down-regulation (3.8×). We studied the expression levels of two additional estrogen-responsive genes in order to further reveal the similarity of the telomeric alcohols to E2. An up-regulation of PDZK1 expression was observed with E2 (41×), with 4-NP (15.2×) as well as with both perfluorinated telomeric alcohols (5.4× with 6:2 FTOH and 2.4× with 8:2 FTOH).

Significant down-regulation of ERBB2 was observed with E2 (4.5×) and 4-NP (4.4×), whereas less pronounced but nonetheless significant down-regulations were also observed with 6:2 (2.4×) FTOH and 8:2 FTOH (2.4×). A small down-regulation of ERBB2 was observed with PFOA (1.5×).

Discussion

A range of fluorinated chemicals synthesized during the past few years are promising for various industrial applications (Lehmler 2005). However, because of the persistent nature of these chemicals, monitoring their environmental fate and their ecotoxicological characteristics is especially warranted (Van de Vijver et al. 2003, 2004). Chemicals that are difficult to degrade biologically may bioaccumulate and may affect the health of humans and biota. Disturbance of the endocrine system is one example of the toxic effects that need careful follow-up. Endocrine disruptors may mimic hormones or interfere indirectly with hormonal pathways. Damage caused by these compounds may, in the long term, lead to drastic effects such as decreased reproduction, or perhaps more subtle effects, such as a disturbance of the developmental system resulting in behavioral effects (e.g., learning disorders). Until now, studies that investigated endocrine-disrupting capacities of fluorinated compounds have been difficult to find. In a review describing developmental toxicity of perfluoroalkyl acids, Lau et al. (2004) highlighted the disturbances of the thyroid gland caused by such compounds (e.g., PFOS causing hypothyroxi-nemia). Because thyroid hormones are known...
to regulate brain development, these findings merit further research.

During the present study, the estrogen-like capacities of the fluorinated compounds 6:2 FTOH, 8:2 FTOH, PFOS, PFOA, and PFNA were studied in vitro. We used a combination of three different in vitro assays to demonstrate these findings. First, we used the E-screen assay, a commonly used high-throughput test to detect estrogen-like compounds in environmental samples. Using this assay, we found that 6:2 FTOH and 8:2 FTOH behave like xenoestrogens in vitro. These compounds clearly induce cell proliferation at 10 µM, the concentration at which many other xenoestrogens are also active (Soto et al. 1995). To complement and corroborate our E-screen results, we studied cell cycle dynamics using flow cytometry. Cells in estrogen-free growth medium do not enter the S-phase easily (Villalobos et al. 1995).

Although > 80% of the MCF-7 cells were in growth arrest (G0/G1-phase of the cell cycle), the addition of xenoestrogens stimulated cells to synthesize new DNA in preparation of cell division, as revealed by the significant increase of the number of cells in the S-phase of the cell cycle. These increases were clearly observable after 24 hr exposure to the telomeric alcohols. Upon comparison of the E-screen assay with flow cytometric analyses, we found similar effective concentrations of E2, 4-NP, and the fluorotelomer alcohols. However, the estrogen-like compounds only induced 2- to 3-fold increases of cell numbers during the E-screen assay, whereas during flow cytometric analyses, we observed up to 5-fold increases of cells in S-phase. Because the exposure periods of E-screen (6 days) and flow cytometric analyses (24 hr) are very different, we also studied cell cycle dynamics after longer exposure periods. After 48 hr, we observed a significant drop of the percentage of cells in S-phase (results not shown). Apparently, cells that are boosted to reenter the cell cycle by a 24-hr xenoestrogen exposure rapidly return to a more modest proliferation rate after 48 hr. One possible explanation is based on the fact that MCF-7 cells express the estrogen receptor as well as the progesterone receptor. Cross-talk exists between nuclear receptors. For instance, progesterone receptor A may act as a repressor of transcriptional activities of different other members of the nuclear receptor family, among them the estrogen receptor (Kraus et al. 1995, 1997). A variation in parameters, such as the ratio of progesterone receptor A to progesterone receptor B, may be the consequence of exposures to (xeno-)estrogens, and apparently this altered ratio may dramatically affect estrogen receptor signaling activities. Another important issue to investigate further is the fact that nuclear receptor levels differ in different

Figure 2. Histograms of DNA content showing the effects of perfluorinated compounds on cell cycle distribution. (A) 0.1% DMSO (solvent control). Cells were cultured in DMEM plus 5% CSFS for 72 hr before exposing them to estrogenic compounds (B, 1 nM E2; C, 10 µM 4-NP; D, 30 µM 6:2 FTOH; and E, 10 µM 8:2 FTOH) and non-estrogenic perfluorinated compounds (F, 50 µM PFOS; G, 50 µM PFNA) for 24 hr. 4-NP (C) was the positive control, and 10 nM TCDD (H) was the negative control.
breast cancer cell lines and even within different clones of a cell line, which may explain why xenoestrogens provoke different cell proliferation responses with different MCF-7 cell lines (Coser et al. 2003; Villalobos et al. 1995).

In order to unravel the mode of action of estrogens and xenoestrogens, gene expression analysis of selected estrogen-responsive genes was performed (Frasor et al. 2003; Inoue et al. 2002). The expression changes of a small number of estradiol-responsive genes such as TFF1, PGR, ESR1, PDZK1, and ERBB2 were studied using reverse-transcription PCR. TFF1 is generally accepted as one of the most reliable estrogen-responsive biomarker genes for in vitro MCF-7 breast cancer cells (Jorgensen et al. 2000; Olsen et al. 2003; Wang and Lou 2004). This factor, also known as pS2, belongs to a family of “trefoil peptides” probably involved in the regulation of cell proliferation. PDZK1 is another frequently reported estrogen-responsive gene (Ghosh et al. 2000; Yoshida et al. 2004). Proteins containing the PDZ domain are involved in organizing cell membrane proteins and are also involved in linking transmembrane proteins to the actin cytoskeleton (Yang et al. 1998). The induction of PDZK1 by E2 is suggested to play a crucial role in membrane alterations that happen upon estrogen treatment such as formation of microvilli. ERBB2 is a transmembrane tyrosine kinase receptor playing a role in mammary oncogenesis. This receptor is up-regulated in MCF-7 cells grown in estradiol-free medium and is down-regulated again upon addition of E2 (Martin et al. 2004; Vendrell et al. 2004). The estrogen-responsive genes TFF1, PGR, PDZK1, and ERBB2 were commonly responsive to E2 as well as to the xenoestrogen 4-NP and the tested fluoro- telomer alcohols. However, although a common up- or down-regulation is observed, the degree of response of the different genes may differ markedly, probably as a consequence of structural differences of the xenoestrogens (Terasaka et al. 2004). These differences may be responsible for and reflect the modes of action. Such differences were also found during the present study. For instance, although 4-NP appears to be a weaker inducer of TFF1 and PGR than 6:2 FTOH, it seems to be a stronger inducer of PDZK1. To discriminate between different xenoestrogens with different modes of action, many more estrogen-responsive genes should be studied. Microarray analyses are used to characterize and classify known and newly detected xenoestrogens according to their different modes of action. The MCF-7 cell line may be an attractive model for this kind of study, due to possible cross-talk between the different hormone receptors of this cell line (Lange et al. 2005). Although we observed a down-regulation of ESR1 expression by E2 and 4-NP, the fluoro- telomer alcohols used in the present study caused a significant, approximately 2-fold up-regulation of this receptor. This finding suggests an alternative mode of action, different from that of the reference compound E2. Up-regulation of the estrogen receptor by presumed xenoestrogens is not unusual, as previously demonstrated for endosulfan, toxaphene, and dieldrin (Soto et al. 1995).

These results warrant further work toward in vivo testing for specific endocrine-disruptive end points. Our results have been generated

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**Table 2. Results of cell cycle analyses of MCF-7 cells exposed to different perfluorinated compounds given as the percentage of cells by phase.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G1/G0-phase</th>
<th>S-phase</th>
<th>G2/M-phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO</td>
<td>90.43 ± 0.13</td>
<td>6.00 ± 1.00</td>
<td>3.57 ± 0.64</td>
</tr>
<tr>
<td>E2 (1 nM)</td>
<td>63.14 ± 1.61</td>
<td>34.84 ± 2.48</td>
<td>2.03 ± 0.48</td>
</tr>
<tr>
<td>4-NP (10 µM)</td>
<td>63.71 ± 1.86</td>
<td>34.64 ± 0.47</td>
<td>1.64 ± 1.41</td>
</tr>
<tr>
<td>6.2 FTOH (30 µM)</td>
<td>49.98 ± 4.09</td>
<td>30.83 ± 3.23</td>
<td>2.19 ± 0.87</td>
</tr>
<tr>
<td>8.2 FTOH (10 µM)</td>
<td>58.83 ± 4.87</td>
<td>39.36 ± 1.78</td>
<td>2.11 ± 0.41</td>
</tr>
<tr>
<td>6:2 FTOH (10 µM)</td>
<td>66.58 ± 1.48</td>
<td>30.83 ± 3.23</td>
<td>2.19 ± 0.87</td>
</tr>
<tr>
<td>PFOS (50 µM)</td>
<td>85.63 ± 0.94</td>
<td>10.49 ± 0.71</td>
<td>3.87 ± 0.73</td>
</tr>
<tr>
<td>PFNA (50 µM)</td>
<td>85.53 ± 1.64</td>
<td>9.89 ± 1.53</td>
<td>4.57 ± 0.42</td>
</tr>
<tr>
<td>PFOA (50 µM)</td>
<td>83.57 ± 1.04</td>
<td>9.17 ± 0.57</td>
<td>6.83 ± 0.59</td>
</tr>
<tr>
<td>TCDD (10 nM)</td>
<td>87.46 ± 0.30</td>
<td>7.96 ± 0.37</td>
<td>4.58 ± 0.46</td>
</tr>
</tbody>
</table>

Values are mean ± SD of three measurements per treatment. During all measurements, coefficient of variation values of the G0/G1 peak were < 3.6 (n = 3).

**Table 3. Results of cell cycle analyses of MCF-7 cells exposed to concentrations of fluoro- telomer alcohols given as the percentage of cells by phase.**

<table>
<thead>
<tr>
<th>Treatment</th>
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</tr>
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<td>66.98 ± 4.09</td>
<td>30.83 ± 3.23</td>
<td>2.19 ± 0.87</td>
</tr>
<tr>
<td>6.2 FTOH (3 µM)</td>
<td>49.98 ± 4.09</td>
<td>30.83 ± 3.23</td>
<td>2.19 ± 0.87</td>
</tr>
<tr>
<td>8.2 FTOH (10 µM)</td>
<td>66.58 ± 1.48</td>
<td>30.83 ± 3.23</td>
<td>2.19 ± 0.87</td>
</tr>
<tr>
<td>6:2 FTOH (10 µM)</td>
<td>66.58 ± 1.48</td>
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Values are mean ± SD of three measurements per treatment. During all measurements, coefficient of variation values of the G0/G1 peak were < 3.6 (n = 3).

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**Figure 3. Effect of perfluorinated chemicals on mRNA expression of estrogen-responsive genes in MCF-7 cells treated with 0.1% DMSO, 1 nM E2, 10 µM 4-NP, 30 µM 6:2 FTOH, 10 µM 8:2 FTOH, 50 µM PFOS, 50 µM PFNA, 50 µM PFOA, or 10 nM TCDD. After exposure to the test compounds for 48 hr, mRNA levels of TFF1 (A), PGR (B), ESR1 (C), PDZK1 (D), and ERBB2 (E) were measured by real-time PCR and normalized using HPRT1 as an internal control. Results are means from three replicate measurements and are expressed as fold relative to 0.1% DMSO; error bars indicate SD.**

*p < 0.05, ** p < 0.001.
with an in vitro system using a single cell line, confirming the estrogen-like properties at different molecular levels. However, at present, it is not at all clear whether fluorotelomer alcohols are causing endocrine disruption under realistic environmental exposure conditions. Information concerning in vivo studies is just becoming available. A one-generation reproductive toxicity study with rats suggests no harmful effect on reproduction (Mylkreest et al. 2005). These authors did not observe any test-substance–related effects on estrous cycle parameters or sperm morphology, motility, or epididymal sperm counts in the first generation offspring. Mylkreest et al. (2005) detected no clear estrogen-like properties in this rat in vivo study. In another long-term rat exposure study (90 days) using a mixture of fluorotelomer alcohols (at doses ≥ 100 mg/kg/day), Ladic et al. (2005) found a persistent elevation of liver weights and thyroid follicular hyperplasia. One possible explanation for the observed discrepancy between our in vivo results and the few in vivo data might be related to differences in fluorotelomer metabolism between the breast cancer cell line and the in vivo exposure condition. These compounds may be converted in rats to other fluorinated molecules, such as PFOA, and hence, fluorotelomer alcohols used in the present study might act as xenoestrogens on various molecular levels. However, at present, it is questionable whether the fluorotelomer alcohols used in the present study might act as endocrine-disrupting xenostero gens on various organisms that might have different metabolizing capacities. Organisms or individuals with a low fluorotelomer-metabolizing activity might be at risk.

Regarding environmental exposure conditions, few data are available at present. Although fluorotelomer alcohols have been detected in the atmosphere at concentrations up to 135 mg/m³ (Martin et al. 2004), there are presently no records of these compounds in surface water, sediment, or wildlife.

In conclusion, we characterized fluorotelomer alcohols as xenoestrogens in vitro. The structural similarities of these compounds and 4-NP, the reference xenoestrogen, offer a possible explanation why these new compounds may act as ligands for the estrogen receptor (Katzenellenbogen 1995). para-Alkylphenols have been shown to bind fully to the estrogen receptors in a dose-dependent manner, and the interaction of alkylphenols with the receptor became stronger with an increase in the number of alkyl carbons (Tabira et al. 1999). In the present study, 6:2 FTOH was characterized as a stronger xenoestrogen than 8:2 FTOH. It is very likely that the chain length of the alkyl group is the responsible factor.

The characterization of fluorotelomer alcohols as in vitro xenoestrogens demonstrates the need to carefully monitor their environmental distribution and to further investigate the effects of perfurinocoupled compounds on biota.

**REFERENCES**


