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Airborne polychlorinated biphenyls (PCBs) reduce telomerase activity and shorten telomere length in immortal human skin keratinocytes (HaCat)

P.K. Senthilkumar^{*}, A.J. Klingelutz[#], J.A. Jacobus^{*▪}, H. Lehmler^{*+}, L.W. Robertson^{*+}, and G. Ludewig^{*,+,&}

^{*}Interdisciplinary Graduate Program in Human Toxicology, The University of Iowa, Iowa City, IA

[▪]Free Radical and Radiation Biology Program, The University of Iowa, Iowa City, IA

⁺Department of Occupational & Environmental Health, The University of Iowa, Iowa City, IA

[#]Department of Microbiology, The University of Iowa, Iowa City, IA

Abstract

PCBs, a group of 209 individual congeners, are ubiquitous environmental pollutants and classified as probable human carcinogens. One major route of exposure is by inhalation of these industrial compounds, possibly daily from inner city air and/or indoor air in contaminated buildings. Hallmarks of aging and carcinogenesis are changes in telomere length and telomerase activity. We hypothesize that semi-volatile PCBs, like those found in inner city air, are capable of disrupting telomerase activity and altering telomere length. To explore this possibility, we exposed human skin keratinocytes to a synthetic Chicago Airborne Mixture (CAM) of PCBs, or the prominent airborne PCB congeners, PCB28 or PCB52 for up to 48 days and determined telomerase activity, telomere length, cell proliferation, and cell cycle distribution. PCBs 28, 52 and CAM significantly reduced telomerase activity from days 18–48. Telomere length was shortened by PCB52 from day 18 and PCB28 and CAM from days 30 on. All PCBs decreased cell proliferation from day 18; only PCB52 produced a small increase of cells in G0/G1 of the cell cycle. This significant inhibition of telomerase activity and reduction of telomere length by PCB congeners suggest a potential mechanism by which these compounds could lead to accelerated aging and cancer.

Keywords

polychlorinated biphenyls (PCB); telomere; telomerase; cell cycle; air pollution; mixture

1. INTRODUCTION

Polychlorinated Biphenyls (PCBs) are a group of 209 individual congeners with 1 to 10 chlorine atoms attached to a biphenyl ring (WHO, 1993). PCBs were used as coolants in transformers and capacitors, as plasticizers and byproducts in paints, cements and sealants,

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Corresponding author: Gabriele Ludewig, PhD, Associate Professor, Dept Occupational & Environmental Health, The University of Iowa, 100 Oakdale Campus, IREH 214, Iowa City, IA 52242-5000, Phone: (319)335-4650, Fax: (319)335-4290, gabriele-ludewig@uiowa.edu.

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and for many other purposes. Although PCB production was banned in the USA and Europe in the late 1970s because of their toxicity, bioaccumulation and biomagnification (Carpenter, 1998), industrial and building products containing PCB materials are still in use. Also, vaporization of PCBs from landfills, contaminated surface water, and construction materials are sources for outdoor and indoor air contamination (Liebl et al., 2004; Persson et al., 2005; Swackhamer and Armstrong, 1986). In addition, PCBs continue to be byproducts of currently produced paints (Hu and Hornbuckle, 2009; Hu et al., 2008). Outdoor air concentrations in Chicago averaged 1.4 ng PCBs/m³, with 10fold higher levels on hot summer days (Sun et al., 2006). Indoor air concentrations are often several magnitudes higher. Wallace and coworkers reported up to 480 ng PCB/m³ in old public buildings (Wallace et al., 1996). Liebl and coworkers found total PCB concentrations (sum of six indicator congeners, PCB 28, 52, 101, 138, 153, 180) in indoor air samples from a school ranging from 690 to 20,800 ng/m³ (median 2,044 ng/m³). Individual congener levels were 33 ng/m³ for PCB28 and 293 ng/m³ for PCB52 (Liebl et al., 2004).

PCBs are toxic to the immune, reproductive, nervous, and endocrine systems and are classified as probable human carcinogens (ATSDR, 2000). A major difficulty in assessing the inhalation hazard posed by PCBs is that the more volatile congeners are not routinely analyzed in environmental or tissue samples. Thus since only isolated studies have measured mono- and di-chlorinated congeners (Ishikawa et al., 2007), the dataset is far from comprehensive. Moreover, the relatively short biological half-lives of lower chlorinated biphenyls due to rapid biotransformation and excretion, which could result in toxic intermediates, further hampers an exact estimate of exposure (Amaro et al., 1996; Saghir et al., 1999). Besides our incomplete knowledge of their occurrence, we are unaware of their mechanisms of toxicity. Therefore the identification of specific toxic endpoints will greatly augment our understanding of the health risks associated with their exposure.

In the indoor air of schools, the lower chlorinated PCBs 28 (2,4,4'-trichlorobiphenyl) and PCB 52 (2,2',5,5'-tetrachlorobiphenyl) contribute to almost 90% of the sum of six indicator congeners (Gabrio *et al.*, 2000). Workers who removed PCB-containing caulking from buildings were found to be exposed to high concentration of PCBs 28 and 52 (Herrick et al., 2007; Kontsas et al., 2004). A moderate increase in the concentration of PCB 28 in the blood of teachers in contaminated schools was observed (Gabrio *et al.*, 2000). Recently it was reported that PCB 28 and 52 are present at high levels in Chicago air (Sun *et al.*, 2006; Zhao *et al.*, 2009). PCB 28 and PCB 52 are non-dioxin like (NDL) PCB congeners, since their ortho chlorine(s) contribute to a less planar biphenyl structure. There is abundant evidence that NDL PCB congeners have toxic effects on various organ systems (Hansen, 1998). PCB 28 has been shown to suppress apoptosis in rat hepatocytes, a mechanism linked to promotion of liver carcinogenesis (Bohnenberger *et al.*, 2001). PCB 52 was shown to induce DNA damage in cultured human lymphocytes (Sandal *et al.*, 2008) and to induce and promote tumors in rat liver (Espandiani et al., 2003; Preston et al., 1985). Highly interesting is that PCB 52 acted synergistically with PCB 77 with respect to genotoxic and cancer initiating activity (Meisner et al., 1992; Sargent et al., 1991; Sargent et al., 1989). It should be considered, however, that these experiments were done with single/few/short term exposures to high doses/concentrations, a completely different scenario than our daily long term low dose exposure through air and food.

While PCBs are proven rodent and probable human carcinogens, their mechanisms of carcinogenicity are not completely understood. Recent results have shown a strong involvement of telomeres and telomerase in the carcinogenic process (Kim *et al.*, 1994; Wu *et al.*, 2003). Telomeres are structures at the end of chromosomes with 5–10 kb repetitive TTAGGG nucleotide sequence. Telomerase is an enzyme that adds TTAGGG to the chromosomal ends to compensate for the progressive loss of telomeric sequences during

normal DNA replication (Cerni, 2000). Particularly the shortening of telomeres has been associated with the development of cancer (Bailey and Murnane, 2006; Stewart et al., 2002), since short telomeres may lead to genetic instability (Meeker *et al.*, 2004) that ensues when short telomeres fuse, particularly in the context of p53 mutation (Farazi *et al.*, 2006). A mechanism to maintain telomeres is eventually necessary, usually through reactivation of telomerase (Delhommeau *et al.*, 2002). Though telomeres and telomerase play a pivotal role in cancer, to our knowledge, only one study has been published on the effect of PCBs on the telomere complex. This study showed that PCB3-p-benzoquinone, a metabolite of PCB3 (4-monochlorobiphenyl), shortens telomeres (Jacobus *et al.*, 2008), but the mechanism, the consequences, whether individual PCB congeners and mixtures have a similar activity and whether PCBs effect the telomerase enzyme are yet unknown.

Contemporary toxicological studies of PCBs often focus on the biological effects of a single congener. A more accurate model of human exposure is achieved with experiments that use mixtures that simulate environmental exposure, such as the Chicago Air Mixture (CAM). The CAM was prepared by mixing 65% Aroclor 1242 and 35% Aroclor 1254; the resulting mixture resembles the average PCB air profile recorded from 1996 to 2002 in Chicago, Illinois (Zhao et al., 2009). Thus, now an environmentally-relevant mixture of PCBs, similar to the PCBs in urban air which people inhale in daily life is available for experimental studies. We report here that two ubiquitous airborne PCB congeners, PCB 28 and PCB 52, as well as a synthetic PCB mixture resembling our PCB exposure in Inner City air reduce telomerase activity and telomere length in human immortal keratinocytes in culture.

2. MATERIALS AND METHODS

2.1. Materials

All cell culture media and components, i.e. Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin-streptomycin, 0.25% trypsin with EDTA, and Phosphate Buffered Saline (PBS) were obtained from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), resazurin and propidium iodide were purchased from Sigma (St. Louis, MO, USA). Colcemide was bought from Alexis Biochemicals (San Diego, CA, USA). PCR reagents and kits were obtained from Qiagen (Valencia, CA, USA). The PCB Chicago Air Mixture (CAM) was prepared as described (Zhao *et al.*, 2009). PCB 28 (2,4,4'-trichlorobiphenyl) was synthesized by Suzuki coupling of 4-chlorobenzene boronic acid with 2,4-dichloro-bromobenzene (Lehmler and Robertson, 2001). PCB 52 was prepared from 2,2',5,5'-tetrachlorobenzidine as described previously (Mönig *et al.*, 1986). The relatively mild reaction conditions in our synthesis approach are unlikely to produce significant amounts of dioxin-like impurities. The purity of both PCB congeners was greater than 99%, as assayed by gas-chromatography.

2.2. Cell culture and PCB exposure regiment

The human keratinocyte (HaCat) cell line was obtained from Dr. C. Svenson, University of Iowa and maintained in DMEM with 10% FBS and 1% penicillin-streptomycin in a humidified incubator at 37° C with 5% CO₂. This cell line was chosen for our studies, since skin is an important exposure route for airborne environmental toxins like PCBs and HaCaT are a good model for carcinogenesis studies. Like the basal cells in the epidermis, which are the target cells for skin carcinogenesis, they are positive for telomerase activity (Harle-Bachor and Boukamp, 1996). HaCaT is a spontaneously immortalized cell line derived from keratinocytes of histologically normal adult male skin (Boukamp et al., 1988); the cells are non-tumorigenic and work well for telomere length studies (Jacobus et al., 2008; Zhang et al., 2003).

To find a non-toxic dose of PCBs in HaCaT cells, 30,000 cells in 24-well tissue culture plates were incubated with PCBs at concentrations of 2–20 μM for six days with a change of medium with fresh compounds on the third day. Control cultures received solvent (DMSO, 0.05% final concentration) alone. After six days, media were removed and cells were incubated with resazurin (5 μM , dissolved in fresh medium) for two hours and the fluorescence intensity of each well was measured using a microplate reader (TECAN, Switzerland). The fluorescence intensity correlates with the number of living cells in the well, since living cells metabolize non-fluorescent resazurin to the fluorescing resorufin. Data points were measured in triplicate and each experiment was performed twice. Data are given as percent of untreated control.

For all other experiments, 100,000 HaCat cells were seeded in 10 cm diameter Petri dishes and exposed to PCB 28, 52 or CAM at a concentration of 5 μM continuously, with regular passaging, for 48 days. Solvent controls received DMSO (0.05% final concentration) only. Media with test compounds or solvent were changed every three days. Cells were trypsinized, counted, and re-seeded at low density every sixth day. The remaining cells on days 6, 18, 30, 42 and 48 were used to determine telomerase activity, telomere length, and cell growth. Cell viability and cell cycle distribution were measured on days 6, 30 and 48. Cell morphology was monitored throughout the exposure period.

2.3 Measurement of telomerase activity

Telomerase activity was measured following the protocol of Wege and coworkers (Wege *et al.*, 2003). Briefly, after trypsinization and centrifugation, cell pellets were resuspended in cell lysis buffer at 1000 cells/ μl density and incubated on ice for 30 minutes. Aliquots of cell lysate were centrifuged at 16,000 g for 20 minutes at 4° C and 3/4th of the supernatant was aliquoted into new micro centrifuge tubes and used for qPCR. The SYBR Green based qPCR reaction was performed with 100 ng (0.2 μl) of Telomerase Primer (TS) and 50 ng (0.1 μl) of Anchored Return Primer (ACX) (Kim and Wu, 1997), 2.5 μl cell lysate supernatant, 11.25 μl Eppendorf MasterMix SYBR ROX (Fisher Scientific, PA, USA) and 10.95 μl of DNase- and RNase-free water in each well of a microplate. Samples were incubated for 20 min at room temperature and PCR was achieved by incubating the sample at 95° C for 10 min followed by 35 cycles of 95° C for 30 sec and 60° C for 90 sec using an Eppendorf RealPlex Thermal Cycler (Eppendorf, Hamburg, Germany). SYBR green fluorescence was determined using an excitation of ~470 nm by dedicated light emitting diodes in each well, and emission was measurements at 520 nm. The Ct (threshold cycle value) was determined from the semi-log amplification plots (log increase in fluorescence versus cycle number) and compared with the standard curves generated from serial dilutions of cell extracts. Each sample was analyzed in duplicate. Telomerase activity was expressed as the percentage relative to the control sample.

2.4 Analysis of telomere length

DNA from trypsinized cells was isolated using the DNA blood mini kit from Qiagen (Valencia, CA, USA) following manufacturer's protocol. DNA concentrations were determined with a spectrophotometer and PCR reactions were performed following the method of Cawthon (Cawthon, 2002). Briefly, 12 ng of DNA from each sample were added to telomere primer 1 (270 nM) and telomere primer 2 (900 nM) for telomeres (T) PCR or 36B4u (300 nM) and 36B4d (500 nM) for single copy gene (S) PCR, in 4.25 μl of telomere buffer in separate multiwell plates. Plates were sealed with plate sealer (Eppendorf, Hamburg, Germany) and incubated at 95° C for 10 min followed by 18 cycles of 95° C for 15 sec and 54° C for 90 sec for telomere plates or 35 cycles of 95° C for 15 sec and 58° C for 1 min for single gene plates using an Eppendorf RealPlex thermal cycler. The T/S ratio was calculated for each corresponding well pair. The T/S ratio of the experimental sample to

the T/S ratio of the control corresponds to the relative change in telomere length of the experimental sample. Each sample was analyzed in duplicate.

2.5 Determination of cell growth and cell viability

To determine the cell growth, trypsinized cells were collected and counted using a Neubauer hemocytometer. The total number of cells in the PCB-treated groups was plotted against the control group to compare the cell growth between groups.

All collected cells i.e., medium-derived and after trypsinization, were washed with PBS, incubated with propidium iodide (PI, 1 µg/ml), and PI-fluorescence of cells was measured using a Becton Dickinson FACS Calibur flow cytometer with Argon Ion Laser at 488 nm excitation and 585 nm band pass filter. Data from 10,000 cells per sample were used to determine the percentages of PI-positive (non-viable) and PI-negative (viable) cells using the WinMDI software.

2.6 Measurement of cell cycle distribution

Trypsinized cells were washed with PBS and approximately 1×10^6 cells were fixed with 70% ethanol overnight. Ethanol-fixed cells were washed once with PBS, and treated with RNase (100 µg/ml) for thirty minutes and PI (50 µg/ml) for one hour. Cell cycle analysis was performed using a Becton Dickinson FACS Calibur flow cytometer with Argon Ion Laser, 488 nm excitation and 585 nm band pass filter. Data were collected from 10,000 cells in list mode and the percentage of cells in each phase of the cell cycle was analyzed using MODFIT software. In these studies control cultures treated with colcemide (0.2 µg/ml for 7 hrs) were used as positive control.

2.7 Statistical Analysis

All data are expressed as the mean $\pm$ standard deviations of triplicates. The data analysis was performed in SAS software, Version 9.2 of the SAS System for Windows. Responses at different treatments were compared with the reference control. Since these comparisons involve a common control, Dunnett's test was used to adjust for their effect on hypothesis testing. Significance levels of 0.05, 0.01 and 0.001 were used for the Dunnett's test.

3. RESULTS

3.1 PCB exposure produces mild cytotoxicity at high concentrations

The dose-response analysis of cytotoxicity of PCB 28, PCB 52 and the CAM in our nonmalignant human skin keratinocyte cell line, HaCaT, showed no reduction in living cells at 2 and 5 µM concentrations of the PCBs compared to the solvent only control (Figure 1). Only a slight reduction in cells was seen at higher concentrations. All other experiments were therefore performed using a 5 µM concentration.

3.2 PCBs decrease telomerase activity

Telomerase activity was measured on days 6, 18, 30, 42 and 48 of exposure to PCBs. All PCBs significantly reduced telomerase activity when compared to the control from the 18th day on (Figure 2). In the PCB 28 and PCB 52 treatment groups, around 30–35% of telomerase activity was lost starting from day 18 till day 48. CAM treatment produced 20–28% reduction of telomerase activity starting from day 18 to day 48. No effect was seen with solvent alone compared to no treatment. Thus, CAM and both PCBs 28 and 52 caused a significant decrease in telomerase activity.

3.3 PCB exposure shortens telomere length

The telomere length was determined at the same exposure times as telomerase activity. A 20% reduction in mean telomere length on day 18 and 30 and a 40% reduction on day 42 and 48 were observed in PCB 52-treated cells (Figure 3). Approximately 10% decrease in mean telomere length was detectable from days 30 to 48 in PCB 28-treated cells. CAM did not reduce the telomere length to a large extent, other than a slight, but significant reduction on day 30 and an about 5% loss of length on days 42 and 48.

3.4 Cell growth was altered, but no cytotoxicity observed

To see whether the reduction in the telomerase activity affected cell growth, cell proliferation was analyzed by determining the cell number in every sample during each subculturing. Cells in the controls were still subconfluent, proliferating, and showed a normal cell size and morphology throughout the whole exposure time. The total number of cells was about 10.2×10^6 per dish in the controls. Since 100,000 cells had been seeded 144 h earlier, this indicates a population doubling time of about 21.5 hours. Cell numbers per plate were decreased in all PCB-treatment groups starting from day 18 until day 48 (Figure 4, bars). With PCB 52 the cell number was reduced to about 9.0×10^6 (days 18 & 30) and 8.7×10^6 (days 42 & 48). PCB 28 and CAM reduced the cell count to about 9.5×10^6 on day 18 and 9.1×10^6 on day 48. In these treatment groups no significant increase in the percent of dead cells, determined by PI-staining, was observed (Figure 4, symbols). Also, no morphological changes like an increase in cell size, indicative of senescence, or fragmented nuclei, indicative of apoptosis, were observed in any of the PCB-treatment groups. This indicates that the reduction in cell number was due to an increase in the population doubling time to about 22.5 – 23 hours.

3.5 The cell cycle profile was not strongly altered

The cell cycle profile was examined to address whether the reduction in cell number and telomerase activity could be due to an accumulation of cells in a specific cell cycle phase. Flow cytometry analysis of cells on days 6, 30, and 48 showed in the control groups the for this cell line normal cell cycle distribution with about 60% of cells in G0/G1, ~31% in S-phase, and about 8–9% of cells in G2/M (Table 1). With PCB 52-treatment a slight and consistent, but not statistically significant elevation in the percent of cells in S phase and decrease in G0/G1 phase was observed. Only a very small change in G0/G1, which was, however, seen at all 3 time points, was observed in the PCB 28 and CAM groups. The solvent, DMSO, did not cause any changes in the cell cycle profile compared to the no-treatment control group. The spindle poison Colcemide, our positive control, produced the expected increase in G2/M.

4. DISCUSSION

PCBs were shown to be carcinogens in rodents and probable carcinogens in humans (Mayes et al., 1998; Silberhorn et al., 1990; Ward et al., 2009). The issue of the mechanism of carcinogenesis by PCB congeners is of importance since humans are exposed to low, but chronic levels of PCBs from food and indoor and outdoor air. Telomeres and telomerase have been shown to be strongly involved in the carcinogenic process. Telomere shortening may result in genetic instability with corresponding predisposition for cancer and the cellular crisis caused by telomeric dysfunction may end in telomerase re-activation, a hallmark of most cancer cells (Delhommeau et al., 2002; Meeker et al., 2004; Wu et al., 2003). Though telomeres/telomerase play a pivotal role in cancer, only one small study with a metabolite of one of the 209 PCB congeners has attempted to address the effect of PCBs on these (Jacobus *et al.*, 2008). Considering our ubiquitous exposure to PCBs through food and inhalation, this represents a broad gap in our knowledge.

We examined the effects of PCB 28 and PCB 52, two lower chlorinated biphenyls that are commonly found in our air, and CAM, a synthetic PCB mixture, which resembles the average PCB profile found in Chicago air (Zhao *et al.*, 2009) on telomeres and telomerase activity. To mimic the chronic exposure to PCBs, exposure periods of up to 48 days were used. This approach is also consistent with the fact that the steps leading to the development of cancer require a certain amount of time (Hanahan and Weinberg, 2000). A concentration of 5 μ M was selected for these studies, since initial toxicity tests confirmed that this concentration is not cytotoxic, which could have produced non-specific confounding effects on the endpoints studied.

Both PCB congeners and the CAM significantly reduced telomerase activity in human HaCat keratinocytes *in vitro* from day 18 to day 48. To our knowledge this is the first report of such an effect of PCBs on telomerase. The significance of this finding is not understood: telomerase is activated in most cancer cells, thus a reduction in activity looks like an anti-carcinogenic effect. However, it has been shown that the lack of telomerase activity leads to the shortening of telomeres (Blasco *et al.*, 1997) and shortened telomeres produce a very high likelihood of telomere fusions and genomic instability, which is associated with the development of cancer (Artandi, 2003). Moreover, telomerase is also active in normal stem cells and in various other proliferating and regenerating cell types (Flores and Blasco, 2010; Harle-Bachor and Boukamp, 1996; Shay and Wright, 2010). A reduction in these cell types could result in premature failure of the function, maintenance and/or regeneration of tissues, even to cancer, if the lower telomerase activity results in a cellular crisis and chromosome instability.

A regulated cell cycle plays an important role in the maintenance of cellular integrity, while its dysregulation may lead to several disorders, including cancer (Collins *et al.*, 1997; Sherr, 1996). One possible mechanism by which PCBs could cause a reduction in telomerase activity is by interfering with cell cycle progression. Telomerase is repressed in cells that exit the cell cycle, like differentiated tissue cells (Holt *et al.*, 1996). In dividing, immortal cells, like the HaCat cell line, telomerase is highly expressed and active throughout all phases of the cell cycle, but was reported to be at a maximum level in the S-Phase of the cell cycle, lower in G1 and G2/S, and still present to some extent when the cells are withdrawn from the cell cycle (Zhu *et al.*, 1996). Thus an increase of cells in G2/M or G1/G0 phase with a concomitant decrease in cells in S-phase could theoretically result in a reduction of telomerase activity of the culture. Indeed, a small but significant reduction in the number of cells at each subculturing was observed in all PCB-treatment groups. Since no increase in cell death was visible, this pointed towards an increase in population doubling time due to a slower progression and/or accumulation of cells in one or more cell cycle phases. Cell cycle analysis at three different time points (days 6, 30, 48) showed a very small increase in the G0/G1 cell population of PCB 28 (~1.8%) and the mixture CAM (~1.3%) compared to media/solvent controls. Although this arrest or slower passage of some cells in G0/G1 is likely to be partially responsible for the decrease in cell number at subculturing time points, it is unlikely that this small shift in cell cycle distribution could have caused the roughly 30% (PCB 28) and 25% (CAM) reduction in telomerase activity. PCB 52 produced a small increase in the number of cells in S phase (about 0.5, 2.0, and 3.3% on days 6, 30, and 48, respectively) with a decrease in the number of cells in G0/G1 phase, indicating a slower passage through S-phase, since the total cell number in these cultures was reduced. An increase in S-phase would be expected to result in an increase in telomerase activity, definitely not a decrease. Therefore other, so far unidentified mechanisms must have caused the ~35% reduction in telomerase activity in PCB 52-treated cultures.

Lack of telomerase activity has been found to be responsible for shortening of telomeres (Hills and Lansdorp, 2009; Lee *et al.*, 1998). We therefore analyzed if telomere length was

also affected as a consequence of the reduction in telomerase activity. PCB 52 significantly shortened the mean telomere length starting from day 18 till day 48. PCB 28 and CAM also shortened the mean telomere length, by about 10 and 5%, respectively. This is by far less than the effect with PCB 52, which resulted in an almost 40% reduction of telomere length compared to controls. There are several mechanisms that could attribute to the shortening of telomeres, in addition to the reduction in telomerase activity. One of the main factors that were identified is oxidative stress. Telomeres, a triple-G-containing structure, are highly sensitive to damage by oxidative stress (Henle *et al.*, 1999; Oikawa and Kawanishi, 1999). Oxidations of these telomeric repeats make the telomeric ends more susceptible to breaks and enhance the rate of telomere attrition (von Zglinicki, 2002). PCBs have been shown to cause DNA damage by oxidative stress (Srinivasan *et al.*, 2001). PCB 52 has been shown to be a stronger producer of oxidative stress than PCB 28 (Lee *et al.*, 2004; Lin *et al.*, 2009; Twaroski *et al.*, 2001). This might therefore be one of the reasons for the more pronounced shortening of mean telomere length in PCB 52-compared to PCB 28- and CAM-treated cells. This shortening of telomere length could lead to chromosomal abnormalities, ploidy changes, and thereby amplification and or/deletion of oncogene and tumor suppressor genes resulting in tumorigenesis (Desmaze *et al.*, 2003; O'Hagan *et al.*, 2002).

It is interesting to note that PCB 28 and PCB 52 although causing the same level of reduction in telomerase activity, seem to act (partially) through different mechanisms, as manifested in different cell cycle effects and considerably stronger reduction in telomere length by PCB 52. Similarly Hamers and coworkers, who screened 24 different PCB congeners in a large battery of *in vitro* toxicity tests, observed clear differences in the profile of PCB 52 and PCB 28 (Hamers *et al.*, 2010). Also, although both PCBs are non-dioxin-like congeners they produce very different changes in gene regulation, i.e. PCB 52 is a CAR-agonist, whereas PCB 28 is an ER agonist; in male mice PCB 52 significantly altered the expression of about 95 genes, whereas PCB 28 affected nearly 600 genes (Heimeier *et al.*, 2010). Obviously the mechanism(s) leading to the observed changes in telomere length could be very complex. Another interesting aspect of our studies is the fact that PCBs 28 and 52 make up only 3.43% and 4.56% by weight of the CAM (Zhao *et al.*, 2009). Thus by far the largest portion of the effects on telomeres and telomerase activity seen with CAM has to be derived from other PCB congeners in this mixture. It could be hypothesized that dioxin-like impurities in CAM may be the driving force for the observed effects. The TEQ of CAM is 2.7 ng/mg mixture (Zhao *et al.*, 2010). Dioxin-like impurities would be in the pM concentration range. Although we do not have data to reject this possibility we believe that it is highly unlikely that an aryl hydrocarbon receptor-based mechanism is the major cause for the effects seen with these congeners or CAM. These findings point out that more studies with more individual PCB congeners are needed to elucidate the mechanism(s) of action and structure-activity relationships for these semi-volatile PCB congeners and to provide the necessary data that would allow *in silico* analysis of mixtures with different PCB congener composition.

We have shown here for the first time that the continuous exposure to an environmentally-relevant mixture of PCBs decreases telomerase activity and shortens telomere length in human cells *in vitro*. These experiments were done at relatively high concentrations that do not reflect current exposure levels of the general population. However, considering the multitude of untested contaminants in our air and our food, we have to question whether the daily exposure to these mixtures, of which PCBs are only a small part, could lead to premature senescence and genomic instability in the cells of our bodies.

Abbreviations

PCB28	(2,4,4'-trichloro biphenyl)
PCB52	(2,2',5,5'-tetrachloro biphenyl)

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Figure 1.

Viable cells per well after 6 days exposure to PCB 28, PCB 52, CAM, or solvent alone. PCB treatments had a small effect only at high, 10 and 20 μ M, concentrations. The height of error bars denotes one SD. Significance code: 0.05 “*”; 0.01 “**”; 0.001 “***”

Figure 2.

Telomerase activity in control and PCB 28-, 52- and CAM-treated HaCat cells. All PCBs significantly reduced telomerase activity from day 18 to 48. No reduction was found on day 6 with either of the PCBs or with solvent alone. The height of error bars denotes one SD. Significance code: 0.05 “*”; 0.01 “**”; 0.001 “***”

Figure 3.

Telomere length in control and PCB 28, 52 and CAM-treated HaCat cells. PCB 52 produced a significant shortening of telomere length from day 18 to 48. PCB 28 and CAM produced shortened telomeres from day 18 on, but to a lesser extent than PCB 52. No effect was seen in solvent-treated cultures. The height of error bars denotes one SD. Significance code: 0.05 “*”; 0.01 “**”; 0.001 “***”

Figure 4.

Cell growth of control cells and PCB 28, 52 and CAM-treated HaCat cells. All PCBs caused a significant reduction in cell growth from day 18 till 48 (bars), but no change in cell viability (symbols and lines) was observed in either of the groups throughout the experiment. The height of error bars denotes one SD. Significance code: 0.05 “*”; 0.01 “**”; 0.001 “***”

Table 1
Effect of PCB 28, 52 and CAM on cell cycle distribution in HaCat cells

PCB 52 produced an increase in S-phase with concomitant decrease in G0/G1 phase at all 3 time points. A mild elevation in G0/G1 phase was observed at all time points in PCB 28- and CAM-treated cells. Col:Colcemide

	Day 6						Day 30						Day 48					
	Medi a	DMS O	CA M	PCB2 8	PCB5 2	Col	Medi a	DMS O	CA M	PCB2 8	PCB5 2	Col	Medi a	DMS O	CA M	PCB2 8	PCB5 2	Col
% of cells in																		
G0/G1	60.5	59.5	61	62	58	3.2	60.3	60.1	61.9	62.0	57.9	3.2	60.2	60.3	61.6	62.0	56.6	35
S	31.3	32.1	30.2	30.1	32.2	60.5	31.2	30.9	30.1	30.0	33.1	60.5	30.9	30.7	30.1	30.1	34.1	46.2
G2/M	8.2	8.3	7.8	7.9	8.8	36.3	8.5	9	8.0	8.0	9.0	36.3	8.9	9	8.3	7.9	9.3	18.8