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Studying placental transfer of highly purified non-dioxin-like PCBs in two models of the placental barrier

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ABSTRACT

Currently, toxicology and toxicokinetics of purified non-dioxin-like polychlorinated biphenyls (NDL-PCBs) are poorly characterised. Transplacental kinetics of NDL-PCBs can be studied in a variety of models, but careful validation of each model is crucial. We aimed to develop a standard operating procedure for establishing an *in vitro* model of the human placental barrier. Using this model, we sought to investigate placental transport kinetics of two NDL-PCB congeners. Firstly, we compared the BeWo cell line of the American Type Culture Collection with the BeWo b30 clone and determined parameters for monolayer formation. Secondly, we performed placental perfusions to validate the *in vitro* model. To that end, the transport of radiolabelled PCB52 and 180 was investigated in both models.

We were not able to grow the ATCC cell line to confluency, but determined monolayer formation using BeWo b30. A confluent monolayer is present by day 4 post-seeding, transepithelial electrical resistance being 44.65 ± 11.06 Ω cm^2 and sodium fluorescein transport being 4.1% ± 0.18. Both measures can be used as indicators for monolayer formation. Results from kinetic studies *in vitro* and *ex vivo* were in excellent agreement. Both NDL-PCBs crossed the placental barrier within 2.5 h. We found PCB180 to transfer more rapidly and PCB52 to associate more with placental tissue. Since transport and association patterns were similar *in vitro* and *ex vivo*, we conclude that the protocol provided here forms the basis for a good model of the placental barrier using BeWo b30. We hypothesise that the observed differences in transport and association patterns of NDL-PCBs may indicate that toxic effects of PCB52 play a more important role regarding placental function, whereas PCB180 may be of greater importance for fetal toxicity.

1. Introduction

The developing fetus represents one of the most vulnerable groups of the human population. Therefore, the placenta plays an important role, serving as a protective barrier as well as facilitating the maternal-fetal exchange of vital compounds. The relative permeability of the placenta is of concern regarding potentially toxic agents consumed by the mother either consciously (e.g., drugs, alcohol) or unconsciously (e.g., environmental pollutants). Polychlorinated biphenyls (PCBs) are amongst the world's most dominant environmental pollutants. PCB levels in humans have decreased within the past 30 years [1] due to a production ban imposed in many countries during the 1970s. However, because of their biological persistence and their tendency to bioaccumulate in the food chain, the human population is still exposed to PCBs mainly via animal based foods such as meat, dairy and fish [2,3]. Of the 209 PCB congeners, the non-dioxin-like PCBs (NDL-PCBs) constitute the major proportion found in the environment and human tissue. Several recent epidemiological studies confirm the presence of NDL-PCBs in pregnant women [4–6]. NDL-PCB concentrations have been reported in maternal and cord blood, suggesting placental transfer of these substances [7,8].

Several models are available to investigate placental transport of PCBs in detail. The use of animal models allows the assessment of uptake through different exposure routes, and biokinetics can be determined for adult and fetal organs. However, careful selection of

Abbreviations: NDL-PCBs, non-dioxin-like polychlorinated biphenyls; ATCC, American type culture collection; Na-Flu, sodium fluorescein; DAPI, 4′,6-diamidino-2′-phenylindole; TEER, Transepithelial electrical resistance; ps, post-seeding; CPM, Counts per minute; P_e/P_{app}, Permeability coefficient/apparent permeability coefficient; FM ratio, Fetal-maternal ratio; DMSO, Dimethyl sulfoxide.

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the model organism is required due to the variability of placental structure among mammals, as well as the length of gestation. Primate placentae are most similar to human [9], but primates are rarely used as a model organism for placental transfer due to a long gestational period, cost and ethical concerns. Guinea pigs have been used as model organisms due to similarities in placental structure and a convenient gestational period [10,11]. However, species-related differences still remain. The *ex vivo* perfusion of the human placenta can be considered a suitable model of direct human relevance. It has the advantages of maintaining the complexity of the whole organ and being easily available. However, this model is technically challenging and unsuitable as a rapid screening tool due to time consuming preparation and the difficulty of obtaining suitable specimens [12]. In contrast, *in vitro* models of the placental barrier including primary cytotrophoblasts isolated from human placentae and choriocarcinoma cell lines, are easy to handle and can be used for rapid screening of placental transfer of a variety of substances over extended exposure periods. Whilst it is more difficult to grow primary placental cells to confluent monolayers [13], choriocarcinoma cell lines like JEG-3, JAr and BeWo can form monolayers when grown on permeable membranes. The BeWo cell line is an established model of the placental barrier [14], which resembles the normal, undifferentiated cytotrophoblast [13]. Although a variety of authors have used BeWo cells in transport studies, the experimental design and conditions vary considerably from study to study [15]. It has been highlighted that a number of factors need to be taken into account when using cell lines for transport experiments, including culture conditions, seeding density and choice of permeable membrane type [16]. Therefore, it is of great importance to establish a standard protocol to guarantee the quality, reproducibility and comparability of results obtained across laboratories. This will ultimately improve the relevance of the obtained kinetic data and make them more meaningful for human exposure assessments.

The b30 clone of BeWo is a single cell derived clone of the ATCC parent BeWo line, developed by Prof. Alan Schwartz and co-workers in the 1980s. In the first place, this was done to ensure that work was carried out on a uniform cell line. In later studies however, it has been observed that the b30 clone has better monolayer formation capacity than the parent cell line [17].

In this study, we developed an *in vitro* model of the placental barrier using BeWo b30 cells, providing a protocol and parameters for assessing monolayer formation. To support the validity of this model, we compared the transfer of highly purified NDL-PCB52 and 180 *in vitro* and in the *ex vivo* perfused human placenta. These congeners are amongst the 6 most abundant NDL-PCBs present in food and are indicators for different PCB patterns in a variety of sample matrices [18]. We chose PCB52 as a representative for a low chlorinated and PCB180 as a representative for high chlorinated PCBs.

2. Materials and methods

2.1. Reagents and chemicals

Highly purified (>99.9999%) 2,2′,5,5′-Tetrachlorobiphenyl (PCB52) and 2,2′,3,4,4′,5,5′-Heptachlorobiphenyl (PCB180) were purchased from Chiron AS and radiolabelled with Carbon-14 (^{14}C) by Blychem Ltd. ^{14}C-PCB52 and ^{14}C-PCB180 were supplied at 0.4 mCi/ml in DMSO (compare Table 1 for physicochemical properties of both NDL-PCBs). Cell culture medium and supplements, trypsin-EDTA, sodium fluorescein solution (Na-Flu), phosphate buffered saline (PBS), paraformaldehyde and Triton-X-100 were purchased from Sigma–Aldrich. Samples from *in vitro* experiments were analysed using Ultima Gold™ scintillation fluid and blank inserts were solubilised with Solvable™, both obtained from PerkinElmer LAS UK Ltd. For sample analysis from *ex vivo* perfusions, Ecosquint scintillation liquid from BN Instruments A/S was used. Primary polyclonal antibodies for tight junction staining of occludin and ZO-1 were rabbit anti-human occludin and rabbit anti-human ZO-1 from Abcam. The secondary antibody was Alexa Fluor® 488 donkey anti-rabbit from Invitrogen. Vectashield mounting medium with DAPI was obtained from Vector Laboratories Ltd.

Table 1
Physicochemical properties of PCB52 and PCB180 according to NLM chemical database (http://chem.sis.nlm.nih.gov/chemidplus/) and distribution of PCBs bound to plasma proteins according to [33].

	PCB52	PCB180
Molecular weight [g/mol]	291.99	395.32
Aqueous solubility [mg/l]	0.0153	0.0039
log *P* (octanol-water)	6.09	8.27
Binding to plasma proteins	~1%	~22%

2.2. Cell culture

The placental choriocarcinoma cell line BeWo b30 was obtained from Prof Harry McArdle (Rowett Research Institute, UK) with the kind permission of Dr. Alan Schwartz (Washington University, St. Louis, MO). The b30 cell lineage of the cells had been authenticated by the European Cell and Culture Collection [19]. The ATCC BeWo cell line was purchased from the European Collection of Cell Cultures (cat #86082803). Cells were cultured in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM F-12 Ham) with phenol red, supplemented with 1% L-Glutamine-Penicillin-Streptomycin, 1% Amphotericin B and 10% fetal bovine serum. All experiments were conducted using this supplemented medium, unless otherwise stated. Cells were routinely maintained in polystyrene cell-culture flasks at 37 °C in 5% CO_2 humidified atmosphere. At confluence, cells were sub-cultured using a 0.4% trypsin-EDTA solution.

2.3. Model optimisation and monolayer confirmation

Transwell® plates (polyester membrane, 0.4 μm pore size) were obtained from Appleton Woods Ltd. Both BeWo cell lines were seeded at 10^5 cells/cm^2 on Transwell® inserts, pre-soaked in medium for 45 min. Medium was changed daily from day 2 post-seeding (ps) until day 13 ps. Transepithelial electrical resistance (TEER) was measured daily from day 2 ps onwards with an Endohm 12 chamber and voltohmmeter (EVOM; World Precision Instruments Inc.). TEER values for the cell layer were obtained by subtracting the intrinsic resistance from the total resistance. TEER values were corrected for surface area and expressed as Ω cm^2. Growth of the cell layer was also monitored visually under the light microscope. In the second phase of model optimisation, BeWo b30 cells were seeded as described above. Cells were left to adhere for two days and medium was changed on days 3, 4 and 5 ps. Development of the monolayer was monitored using TEER measurements, Na-Flu transport and transmission electron microscopy (TEM). Na-Flu transport was conducted by adding 5 μM Na-Flu to the apical chamber and supplemented DMEM F-12 Ham medium without phenol red to the basal chamber. Plates were incubated for 3 h at 37 °C. 50 μl samples were removed from the basal chamber into a black 96-well plate and read at excitation 485 nm and emission 520 nm on a microplate reader (BMG Labtech FLUOstar Optima). Values were blank corrected and sample concentrations were determined using a Na-Flu standard curve. For TEM imaging, cells grown on insert membranes were fixed and processed according to Lahtinen et al. [20]. Samples were imaged on days 3–5 ps. To examine the presence of tight junctions in the monolayer, antibody staining was performed on day 4 ps. Cells grown on insert membranes were fixed in 2% paraformaldehyde and permeabilised with 0.3% Triton-X-100. Cells were incubated with primary antibody against ZO-1 or occludin and washed before incubation with the secondary antibody.

2.4. ^{14}C-PCB transfer and recovery in vitro

To estimate the rate of transfer of ^{14}C-PCB180 and ^{14}C-PCB52, BeWo b30 cells (passage 34–41) were seeded at 10^5 cells/cm^2 on Transwell® inserts as described above. Transfer studies were conducted on day 4 ps at 37 °C and 5% CO_2. Monolayer integrity was evaluated using TEER measurements. TEER values had to fall within a previously determined range in order for the experiment to be carried out. Rate of transfer through the insert membranes alone was also estimated. Solutions of ^{14}C-PCB180 and ^{14}C-PCB52, previously dissolved in Dimethyl sulfoxide (DMSO), were made up at 15 μM (0.5 μCi/ml) in medium. The total concentration of DMSO in the medium was 0.125% (v/v). Previous experiments in our lab showed that neither PCB52 nor PCB180 decreased cell viability or disrupted tight junctions of BeWo cells at this concentration and using DMSO as a vehicle. ^{14}C-PCB solutions were added to the apical and medium to the basal chambers chambers of the Transwell®. Experiments were carried out over two time courses, a short (2 h) and a long time course (24 h). During the short time course, three 50 μL aliquots were taken from the apical and from the basal chamber after 30, 60, 90 and 120 min. The same procedure was performed during the long time course after 2, 4, 6 and 24 h. Three separate wells were used for each time point and were discarded after sampling to avoid alterations to the concentration gradient between apical and basal chambers (no sampling and replacing). The amount of ^{14}C-PCB180 or ^{14}C-PCB52, respectively, was determined