3,3′,4,4′,5-Pentachlorobiphenyl Inhibits Drug Efflux Through P-Glycoprotein in KB-3 Cells Expressing Mutant Human P-Glycoprotein

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The effects on the drug efflux of 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126), the most toxic of all coplanar polychlorinated biphenyls (Co-PCBs), were examined in KB-3 cells expressing human wild-type and mutant P-glycoprotein in which the 61st amino acid was substituted for serine or phenylalanine (KB3-Phe61). In the cells expressing P-glycoproteins, accumulations of vinblastine and colchicine decreased from 85% to 92% and from 62% to 91%, respectively, and the drug tolerances for these chemicals were increased. In KB3-Phe61, the decreases in drug accumulation were inhibited by adding PCB-126 in a way similar to that with cyclosporine A: by adding 1 µM PCB-126, the accumulations of vinblastine and colchicine increased up to 3.3- and 2.3-fold, respectively. It is suggested that PCB-126 decreased the drug efflux by inhibiting the P-glycoprotein in KB3-Phe61. Since there were various P-glycoproteins and many congeners of Co-PCBs, this inhibition has to be considered a new cause of the toxic effects of Co-PCBs.

INTRODUCTION

Among the group of polychlorinated biphenyls (PCBs), coplanar PCBs (Co-PCBs) are known to be highly toxic and indestructible congeners [1, 2]. Co-PCBs had been produced as useful artificial lubricants for the chemical industry and in the manufacture of a variety of daily-use products until their production and use were prohibited. These chemicals have thoroughly polluted the environment, and may have accumulated via the food chain and bioaccumulation in humans and animals, especially in their lipid-rich organs [2, 3, 4, 5]. In the environment, 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126) which is the most toxic congener of Co-PCBs is also highly polluting the environment [1, 6]. Like dioxin, the mixture of Co-PCBs or the metabolites of Co-PCBs seems to produce either estrogenic or antiestrogenic effects [7, 8]. Co-PCBs were the agonists for arylhydrocarbon receptors (AhR) [1, 9], and they inhibited the estrogenic response via crosstalk between AhR and estrogen receptors [10, 11]. Actually, Co-PCBs have acted as environmental endocrine disruptors, exerting adverse biological effects even at a very low dose [6, 12]. They also disturbed normal fetal and neonatal development, producing malformations and the onset of cancer [4, 6, 13, 14].

Drug transport systems such as P-glycoprotein are present on the cell membrane to exclude toxic xenobiotics [15] and to transport metabolites such as steroid hormones [16]. P-glycoprotein is capable of transporting a variety of structurally unrelated chemicals [17] and can induce multidrug resistance during cancer chemotherapy [18, 19]. The P-glycoprotein-mediated transport of chemicals was inhibited by other chemicals. Cyclosporine A, for example, competed with vinblastine for a site to bind to P-glycoprotein, and inhibited the transport of this substrate [20]. The most hydrophobic steroid hormone, progesterone, blocked vinblastine transport, although progesterone itself was not transported. In a series of steroid hormones, it was revealed that the more hydrophobic the congeners, the more potent their interference with the transport of other chemicals [21]. One of the congeners of Co-PCBs, 3,3′,4,4′-tetrachlorobiphenyl (PCB-77), was not transported by human P-glycoprotein, but accumulated abundantly in cells as can be expected due to its lipophilic nature [22]. Co-PCBs easily enter cells and may accumulate in the cell membrane due to their lipophility. P-glycoprotein in the cell membrane may be exposed to Co-PCBs, which may modify the transport of other drugs and metabolites in the same way as do hydrophobic steroid hormones and cyclosporine A, thus altering the effects of those substances.
Table 1. Drug tolerance for vinblastine and colchicine in KB3-Vec and KB3-MDR1s, and the ratios for the tolerance of KB3-Vec to KB3-MDR1s (KB3-MDR1s/KB3-Vec).

| Drug          | KB3-Vec | KB3-His61 | KB3-Phe61 | KB3-Ser61 |
|---------------|---------|-----------|-----------|-----------|
| Vinblastine (ID50, nM) | 3.4     | 26        | 30        | 40        |
| (KB3-MDR1s/KB3-Vec)     | (1)     | (7.6)     | (8.8)     | (11.8)    |
| Colchicine (ID50, nM)   | 8.8     | 46        | 859       | 43        |
| (KB3-MDR1s/KB3-Vec)     | (1)     | (5.2)     | (97.6)    | (4.9)     |

Note. Inhibition dose for 50% cell growth (ID50) by adding chemicals was expressed as the index of drug tolerance.

Though P-glycoprotein has a low specificity for substrates, it was found to contain many mutations of amino acids that modulated substrate specificity [23, 24]. Such mutations might be involved in the modification of binding and transporting mechanisms. The toxicity of drugs was reduced in the transformant cells expressing P-glycoprotein, probably due to an enhancement of the drug efflux. These drug tolerances varied among the transformant cells in which the His 61 position (His61) in the predicted transmembrane domain 1 of human P-glycoprotein was replaced with a variety of amino acids [24]. Accordingly, the effect of Co-PCBs might differ among the mutants of P-glycoproteins. The possibility of a modification in P-glycoprotein by binding with Co-PCBs must be examined. Here, we investigated the effect of PCB-126 on the efflux of vinblastine and colchicine in KB-3 cells transfected with multidrug resistant (MDR1) genes which code both wild and His61 mutant P-glycoproteins.

**MATERIALS AND METHODS**

**Chemicals**

For radioactive tracers, we used [G- 3H]-vinblastine sulphate (470 GBq/mmol) (Amersham Pharmacia Biotech, NJ, USA) and [3H]-colchicine (2,830 GBq/mmoul) (Perkin Elmer Japan Co Ltd, Tokyo, Japan). As the representative of Co-PCBs, PCB-126 (Kanto Kagaku, Tokyo, Japan) was used, and cyclosporine A (Sigma, Mo, USA) was used as the inhibitor of P-glycoprotein.

**Cell culture**

The transformant cells of human cultured cells, that is, KB3-1 expressing with wild-type human P-glycoprotein (KB3-His61) and the mutant P-glycoprotein, in which His61 was substituted with serine (KB3-Ser61) or phenylalanine (KB3-Phe61), were prepared as reported in [24]. As the control, KB3-1 transfected with the transfection vector pHAMAIRE′sneo (KB3-Vec) was also used. These cells were maintained in medium 199 supplemented with 10% fetal calf serum (FCS) in an atmosphere of 5% CO2 at 37°C, and the transformant cells were maintained in the same medium with appropriate reagents; KB3-His61 and KB3-Ser61 were maintained with 100 µM vinblastine and KB3-Phe61 with 60 µM colchicine.

**Drug tolerance of cells**

The cells were seeded in 96-well microplates at a density of 10^5/well in a 500-µL medium identical to that of the maintenance culture except for varied concentrations of vinblastine and colchicine, and then incubated for 4 days. The viability of these cells was then measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Wako Co Ltd, Osaka, Japan). The inhibition dose for 50% cell growth (ID50) was calculated as the index for drug tolerance.

**Drug accumulation and its inhibition**

For a determination of the cellular accumulation of the chemicals, coverslips with a 24-well multidish (Nalgen Nunc International, Ill, USA) were used. Cells were seeded at a density of 5 × 10^4/well in a 750-µL maintenance medium on the dish and incubated in 5% CO2 at 37°C. After 4 days, the medium was replaced with a fresh medium without colchicine, and the cells were incubated for 6 hours. The medium was again replaced with a 750-µL fresh medium containing either 11 nM [3H]-vinblastine (5.16 kBq/mL) or 11 nM [3H]-colchicine (31.1 kBq/mL). The coverslips were removed from the 24-well dish after a 1-, 2-, and 3-hours incubation. These cells were washed 3 times with PBS, and treated with a lysing solution (cell culture lysis reagent, Promega, Wis, USA) overnight. Radioactivity was then measured by a liquid scintillation counter. The accumulation was indicated as pmol/well.

To examine the effect of PCB-126 on drug accumulation, PCB-126 and the inhibitor of p-glycoprotein, cyclosporine A, were added to the incubation medium. One hundred pM to 10 µM PCB-126 or cyclosporine A was added to the medium, and the accumulations of vinblastine and colchicine were measured.

**Statistics**

The student’s t-test was employed to examine the statistical significance of the accumulation between cell groups.
RESULTS AND DISCUSSION

Table 1 indicates the drug tolerances of KB3-Vec, KB3-His<sup>61</sup>, KB3-Ser<sup>61</sup>, and KB3-Phe<sup>61</sup> in the medium with vinblastine or colchicine. The ID<sub>50</sub> increased in all the cell groups expressing P-glycoprotein (KB3-MDR1s), KB3-His<sup>61</sup>, KB3-Ser<sup>61</sup>, and KB3-Phe<sup>61</sup>, except for KB3-Vec. As for vinblastine, the ID<sub>50</sub> increased around 10-fold in all KB3-MDR1s except for KB3-Vec, and the order of drug tolerances was KB3-Ser<sup>61</sup> > KB3-Phe<sup>61</sup> > KB3-His<sup>61</sup>. With colchicine, the ID<sub>50</sub> increased around 5-fold in KB3-His<sup>61</sup> and KB3-Ser<sup>61</sup>, and 98-fold in KB3-Phe<sup>61</sup>. The increase in the ID<sub>50</sub> for colchicine in KB3-Phe<sup>61</sup> was extremely
high, indicating a special relation between the side chain of Phe$^{61}$ and the relatively smaller substrate, colchicine. Taguchi et al showed an inverse relation between the bulk of the side chain of the 61st amino acid in P-glycoprotein and the molecular weight of the drug; the P-glycoprotein, which has a large side chain in the 61st amino acid, indicated less sensitivity to the smaller molecular weight of the chemicals, and vice versa [24].

Figure 1a shows the vinblastine uptake as a function of time in KB3-Vec and of reductions in the uptake in KB3-MDR1s. The uptakes of vinblastine in KB3-Vec increased hyperbolically during a 3-hour incubation as reported in [22], and the accumulations after that incubation in KB3-His$^{61}$, KB3-Ser$^{61}$, and KB3-Phe$^{61}$ were reduced by 85%, 88%, and 92%, respectively, compared to KB3-Vec. Thus, under such conditions, most of the drug might have been transported to an extracellular site by the P-glycoproteins which were expressed by transfection with each MDR1 gene. These excretions might contribute to the increase in ID$_{50}$ in KB3-MDR1s.

Figures 1b and 1c show the effects of cyclosporine A (b) and PCB-126 (c) on the accumulations of vinblastine after a 3-hours incubation in KB3-Vec and KB3-MDR1s; the ratios between the accumulations with and without cyclosporine A or PCB-126 in each cell group were compared. An increase in the ratios indicates an increased inhibition against the drug efflux due to the addition of cyclosporine A or PCB-126. The ratios were increased by adding more than 1 $\mu$M cyclosporine A in all KB3-MDR1s, but there was no clear increase in the ratio in KB3-Vec. The ratios in KB3-His$^{61}$, KB3-Ser$^{61}$, and KB3-Phe$^{61}$ increased 7.0-, 6.3-, and 7.5-fold, respectively, by adding 1 $\mu$M cyclosporine A, and those accumulations increased 9.2-, 9.1-, and 10.3-fold, respectively, by adding 10 $\mu$M cyclosporine A. Though the increase in the ratio was less than 2-fold by adding 0.1 $\mu$M cyclosporine A, those in KB3-Phe$^{61}$ and KB3-His$^{61}$ were increased significantly. The ratio in KB3-Phe$^{61}$ was relatively higher than in the other cell groups, though the difference among KB3-MDR1s was less than 20%. By adding 0.1, 1 and 10 $\mu$M PCB-126, the ratio in KB3-Phe$^{61}$ increased 1.4-, 3.3-, and 2.8-fold, respectively, thus revealing an inhibition of the drug efflux by the addition of PCB-126. PCB-126 might have had an effect on the mutant P-glycoprotein in KB3-Phe$^{61}$, thus inhibiting the drug efflux. The ratios in KB3-His$^{61}$ and KB3-Ser$^{61}$ also increased around 1.5-fold, but their effect was no different from that in KB3-Vec.

Figure 2a shows the colchicine uptake as a function of time in KB3-Vec and of reductions in the uptakes in KB3-MDR1s. The colchicine uptake displayed a somewhat linear function compared to the vinblastine uptake in all cell groups. The accumulations after a 3-hours incubation in KB3-His$^{61}$, KB3-Ser$^{61}$, and KB3-Phe$^{61}$ were reduced, respectively, by 75, 62, and 91% compared to that in KB3-Vec. Thus, under those conditions, KB3-Phe$^{61}$ demonstrated the highest ability to transport colchicine, while KB3-Ser$^{61}$ showed the lowest.

Figures 2b and 2c show the effects of cyclosporine A (b) and PCB-126 (c) on the accumulations during a 3-hours incubation in KB3-Vec and KB3-MDR1s. The ratios between accumulations with and without these chemicals in KB3-His$^{61}$, KB3-Ser$^{61}$, and KB3-Phe$^{61}$ increased 2.6-, 2.0-, and 5.8-fold by adding 1 $\mu$M cyclosporine A, and increased 3.6-, 2.9-, and 9.2-fold by adding 10 $\mu$M cyclosporine A, respectively, while there was no such effect in KB3-Vec. The cell group which showed the largest reduction in drug accumulation, KB3-Phe$^{61}$, also showed the largest increase in accumulation when the inhibitor for P-glycoprotein was added. This might be due to an inhibition of the efflux through P-glycoprotein as reported in [21]. These effects of cyclosporine A on drug accumulation were considered due to a modification of P-glycoprotein by its binding [20]. By adding 1 and 10 $\mu$M PCB-126, the ratios in KB3-Phe$^{61}$ increased 2.3- and 2.1-fold, respectively, indicating an inhibition of the drug efflux. The ratios in KB3-His$^{61}$ and KB3-Ser$^{61}$ increased slightly, though there was no significant difference from that in KB3-Vec. Thus, PCB-126 might have bound with Phe$^{61}$ mutant P-glycoprotein to inhibit the efflux of colchicine.

In all KB3-MDR1s, accumulations of vinblastine were equally reduced, and cyclosporine A inhibited these reductions to almost the same degree among KB3-MDR1s. However, the reductions and inhibitions in the accumulation of colchicine varied among cell groups. Previously, one of the present authors revealed that the cells expressing mutant P-glycoprotein which possessed a larger 61st amino acid gained a tolerance to various chemicals, whereas, in the cells expressing the mutant P-glycoprotein which possessed a smaller 61st amino acid, the tolerance for large chemicals was superior to that for small chemicals [24]. These results were explained as being due to the difference in the bulk of the side chain of the 61st amino acid in P-glycoprotein, rather than to the electron charge or hydrophilicity in the side chain. In this experiment, KB3-Phe$^{61}$ showed a superior ability for excretion in both vinblastine and colchicine, and the excretion in KB3-Phe$^{61}$ was more potently inhibited by cyclosporine A and PCB-126 compared to the other cells. These results showed that the Phe$^{61}$ mutant P-glycoprotein in which the side chain of the 61st amino acid was larger than that in the other mutants was less specific for substrates. Therefore, it might have reacted to a variety of substrates with almost the same affinity. However, the wild type, His$^{61}$ P-glycoprotein, and Ser$^{61}$ mutant P-glycoprotein were somewhat specific for substrates, and their affinity was predominant in the larger substrate, vinblastine. The Phe$^{61}$ mutant P-glycoprotein might have the ability to bind with a variety of chemicals dissimilar to one another in structure and molecular size. Thus, PCB-126 might also bind to the Phe$^{61}$ mutant P-glycoprotein and inhibit the efflux of vinblastine and colchicine, while being unable to react with the other P-glycoproteins.
The contamination level of Co-PCBs and the concentrations used in this experiment will now be discussed. As a result of their locations on the food chain, several marine mammals showed high levels of contamination with PCBs, that is, up to around 5 mg/kg in their blubber [25]. In humans who consumed high levels of fish, the total content of lipid-adjusted plasma PCBs increased up to 770 µg/kg [26], and PCB-126 accumulated up to 1.4 µg/kg in plasma lipid [4]. Among workers employed in capacitor manufacturing, the concentrations of high- and low-chlorinated PCBs in adipose tissue increased up to 165 and 414 µg/kg, respectively [27]. PCBs are highly lipophilic and may accumulate in the lipid bilayers of the cell membrane. In the present experiment, the effect of Co-PCBs was revealed at levels of 0.1 to 10 µM PCB-126, that is, 30 to 3000 µg/kg of PCB-126. Therefore, in populations exposed to an environment highly contaminated with PCBs, the concentrations of PCBs or Co-PCBs in their cell membranes would probably increase around the concentrations found in this experiment.

In conclusion, it was indicated that PCB-126 inhibited the drug efflux through the mutant P-glycoprotein in KB3-Phe41. The interaction of PCB-126 with P-glycoprotein was modified by the mutation of the 61st amino acid of the protein. There have been many amino acid mutations that modulated substrate specificity in P-glycoprotein [28]. Therefore, there may be P-glycoproteins which are affected by some congener(s) of Co-PCBs as revealed in this experiment. Though the precise mechanism of toxicity is still unknown, P-glycoprotein plays a role both in the extrusion of xenobiotics and in the transport of steroid hormones [16, 21]. Consequently, not only the toxicities of xenobiotics but also the effects of steroid hormones may be disturbed by Co-PCBs. In particular, the regulation of brain differentiation in an embryo by steroid hormones seems to be influenced by Co-PCBs. In addition to the reported mechanism for toxicities [1, 7, 8, 9, 10, 11, 12], the effect of Co-PCBs on P-glycoprotein has to be considered as a new cause of the toxic effects of these chemicals.

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