Table 2. Genes related to the sensitivity to MMC, vinorelbine, paclitaxel, and SN-38 in 42 human cancer cell lines

Rank	Gene	Genbank ID	r	P
A. MMC				
Sensitive				
1	*SF1*	D26121	0.566	0.001
2	*CBR3*	Ab004854	0.486	0.006
3	*EMS1*	M98343	0.480	0.010
4	*JUN*	J04111	0.473	0.015
5	*SFRS9*	U30825	0.448	0.010
6	*NMBR*	M73482	0.428	0.012
7	*RBMX*	Z23064	0.419	0.012
8	*SOD1*	M13267	0.418	0.024
9	*NOL1*	X55504	0.415	0.025
10	*PELP1*	U88153	0.405	0.019
11	*ARHA*	L25080	0.404	0.030
12	*AARS*	D32050	0.398	0.018
13	*NME1*	X17620	0.398	0.032
14	*HNRPA2B1*	M29065	0.390	0.044
15	*NME2*	L16785	0.378	0.025
16	*VAT1*	U18009	0.376	0.031
17	*SERPINB10*	U35459	0.372	0.028
18	*KIAA0436*	AB007896	0.353	0.041
19	*DRPLA*	D31840	0.350	0.049
20	*MC3R*	L06155	0.346	0.049
Resistant				
1	*SPTBN1*	M96803	−0.450	0.013
2	*PET112L*	AF026851	−0.425	0.027
3	*CAPN1*	X04366	−0.421	0.032
4	*MEL*	X56741	−0.414	0.028
5	*PACE*	X17094	−0.380	0.035
6	*DVL2*	AF006012	−0.370	0.034
7	*LOC54543*	AJ011007	−0.366	0.022
8	*PAPOLA*	X76770	−0.351	0.033
9	*RPLP2*	M17887	−0.345	0.049
10	*ARF4L*	L38490	−0.340	0.042
B. Vinorelbine				
Sensitive				
1	*ARHA*	L25080	0.534	0.003
2	*NME2*	L16785	0.521	0.001
3	*VIL2*	X51521	0.463	0.015
4	*YWHAQ*	X56468	0.450	0.011
5	*HK1*	M75126	0.449	0.016
6	*SATB1*	M97287	0.439	0.006
7	*CAMLG*	U18242	0.439	0.007
8	*CARS*	L06845	0.433	0.007
9	*CCNB1*	M25753	0.427	0.013
10	*U2AF1*	M96982	0.424	0.022
11	*PTMA*	M26708	0.423	0.018
12	*MLC1SA*	M31211	0.397	0.022
13	*NME1*	X17620	0.393	0.035
14	*SARS*	X91257	0.386	0.032
15	*CDC20*	U05340	0.385	0.029
16	*PPP4C*	X70218	0.385	0.039
17	*TNFAIP3*	M59465	0.384	0.023
18	*EEF1D*	Z21507	0.384	0.023

NOTE: Column 2 shows the name of the gene according to HUGO database. Column 4 shows Pearson correlation coefficient between chemosensitivity to drugs and gene expression. "Sensitive" indicates candidate genes sensitive to each drug. "Resistant" indicates genes resistant to each drug.

Table 2. Genes related to the sensitivity to MMC, vinorelbine, paclitaxel, and SN-38 in 42 human cancer cell lines (Cont'd)

Rank	Gene	Genbank ID	r	P
19	*PFKP*	D25328	0.365	0.028
20	*ENTPD2*	U91510	0.365	0.037
21	*CCL5*	M21121	0.358	0.035
22	*ACAT1*	D90228	0.352	0.048
23	*IQGAP1*	L33075	0.351	0.042
24	*PAX5*	M96944	0.342	0.038
25	*NRGN*	Y09689	0.336	0.042
26	*K-α-1*	K00558	0.328	0.048
27	*NDUFB7*	M33374	0.321	0.049
Resistant				
1	*HOXB1*	X16666	−0.600	0.000
2	*F10*	K03194	−0.514	0.002
3	*GPX2*	X53463	−0.509	0.002
4	*NR1I2*	AF061056	−0.498	0.002
5	*ANXA4*	M19383	−0.481	0.005
6	*PDLIM1*	U90878	−0.465	0.006
7	*LIPC*	X07228	−0.464	0.004
8	*SERPINF2*	D00174	−0.447	0.004
9	*HSD17B1*	M36263	−0.443	0.014
10	*MAN2B1*	U60266	−0.440	0.008
11	*LSS*	D63807	−0.430	0.014
12	*PIK3CG*	X83368	−0.415	0.010
13	*DBN1*	U00802	−0.414	0.017
14	*NDUFA4*	U94586	−0.410	0.038
15	*BDH*	M93107	−0.399	0.024
16	*BCL2L1*	Z23115	−0.385	0.039
17	*EEF1B2*	X60656	−0.383	0.030
18	*F2*	V00595	−0.382	0.026
19	*RARA*	X06614	−0.369	0.029
20	*ITGB4*	X53587	−0.367	0.042
21	*IMPA1*	X66922	−0.367	0.042
22	*PACE*	X17094	−0.367	0.042
23	*AGA*	M64073	−0.361	0.042
24	*MVD*	U49260	−0.353	0.038
25	*EHHADH*	L07077	−0.346	0.039
26	*TFPI2*	D29992	−0.343	0.035
27	*MARCKS*	M68956	−0.342	0.045
28	*FGB*	J00129	−0.334	0.035
29	*GPD1*	L34041	−0.322	0.049
C. Paclitaxel				
Sensitive				
1	*ADH6*	M68895	0.513	0.002
2	*RAB28*	X94703	0.480	0.007
3	*U2AF1*	M96982	0.441	0.017
4	*GPC1*	X54232	0.440	0.013
5	*HK1*	M75126	0.439	0.020
6	*CARS*	L06845	0.436	0.006
7	*TNFAIP3*	M59465	0.433	0.009
8	*K-α-1*	K00558	0.418	0.010
9	*PFKP*	D25328	0.416	0.012
10	*GDI2*	D13988	0.411	0.033
11	*VIL2*	X51521	0.410	0.034
12	*RUNX2*	AF001450	0.409	0.038
13	*NME2*	L16785	0.407	0.015
14	*CDC20*	U05340	0.395	0.025
15	*GNAI2*	X04828	0.391	0.033

(Continued on the following page)

Table 2. Genes related to the sensitivity to MMC, vinorelbine, paclitaxel, and SN-38 in 42 human cancer cell lines (Cont'd)

Rank	Gene	Genbank ID	r	P
16	*ARHA*	L25080	0.381	0.041
17	*CNR2*	X74328	0.378	0.030
18	*PPP2R2B*	M64930	0.376	0.026
19	*SLC6A8*	L31409	0.374	0.046
20	*DDX9*	L13848	0.374	0.042
21	*ACAT1*	D90228	0.369	0.038
22	*PI3*	Z18538	0.329	0.047
Resistant				
1	*NAP1L1*	M86667	−0.530	0.004
2	*HOXB1*	X16666	−0.516	0.004
3	*PACE*	X17094	−0.507	0.004
4	*MAN2B1*	U60266	−0.486	0.003
5	*GPX2*	X53463	−0.480	0.004
6	*DBN1*	U00802	−0.469	0.006
7	*ANXA4*	M19383	−0.468	0.007
8	*SERPINF2*	D00174	−0.463	0.003
9	*AGA*	M64073	−0.444	0.011
10	*BCL2L1*	Z23115	−0.428	0.021
11	*LIPC*	X07228	−0.401	0.015
12	*BDH*	M93107	−0.393	0.026
13	*LSS*	D63807	−0.384	0.030
14	*PDLIM1*	U90878	−0.372	0.033
15	*ZNF161*	D28118	−0.368	0.038
16	*UBE2E1*	X92963	−0.363	0.032
17	*TLE1*	M99435	−0.360	0.039
18	*RARA*	X06614	−0.359	0.034
19	*PTPRN*	L18983	−0.357	0.035
20	*APOE*	M12529	−0.353	0.048
21	*F10*	K03194	−0.348	0.040
22	*NR1I2*	AF061056	−0.342	0.041
23	*UBE2L3*	X92962	−0.332	0.045
24	*FGB*	J00129	−0.313	0.049
D. SN-38				
Sensitive				
1	*EMS1*	M98343	0.573	0.001
2	*JUN*	J04111	0.564	0.003
3	*IL-6*	X04602	0.514	0.003
4	*RPL23*	X52839	0.495	0.004
5	*CDKN3*	L25876	0.455	0.017
6	*RPL3*	X73460	0.445	0.011
7	*TFPI*	J03225	0.442	0.009
8	*MRPL3*	X06323	0.437	0.009
9	*HLA-C*	M11886	0.424	0.014
10	*AARS*	D32050	0.419	0.012
11	*ARHGDIA*	X69550	0.416	0.031
12	*NOL1*	X55504	0.406	0.029
13	*SF1*	D26121	0.394	0.031
14	*SOD1*	M13267	0.389	0.037
15	*VEGF*	M32977	0.384	0.043
16	*EIF2S1*	J02645	0.382	0.034
17	*CDH5*	X79981	0.372	0.030
18	*FOSL1*	X16707	0.371	0.047
19	*IDS*	M58342	0.366	0.047
20	*PMVK*	L77213	0.364	0.044
21	*PPP2CB*	X12656	0.364	0.041
22	*NMBR*	M73482	0.362	0.035

(*Continued*)

Table 2. Genes related to the sensitivity to MMC, vinorelbine, paclitaxel, and SN-38 in 42 human cancer cell lines (Cont'd)

Rank	Gene	Genbank ID	r	P
23	*RPL26*	X69392	0.358	0.035
24	*PELP1*	U88153	0.356	0.042
25	*MC3R*	L06155	0.356	0.042
26	*RPS8*	X67247	0.355	0.036
Resistant				
1	*CAPN1*	X04366	−0.496	0.010
2	*MEL*	X56741	−0.478	0.010
3	*PACE*	X17094	−0.443	0.012
4	*TIMP2*	J05593	−0.433	0.019
5	*AOP2*	D14662	−0.422	0.025
6	*ZNF174*	U31248	−0.402	0.018
7	*ID3*	X69111	−0.393	0.038
8	*KLF5*	D14520	−0.384	0.036
9	*CALD1*	M64110	−0.382	0.031
10	*LOC54543*	AJ011007	−0.368	0.021
11	*PTPN3*	M64572	−0.363	0.038
12	*ACTB*	X00351	−0.362	0.025
13	*LY6E*	U42376	−0.360	0.037
14	*ID1*	D13889	−0.343	0.044

was >90% as evaluated by transfection of a plasmid expressing the enhanced green fluorescent protein (data not shown). To validate this screening system, we examined the effect of *NQO1* gene, coding DT-diaphorase that increases cellular sensitivity to MMC (12). As shown in Fig. 3B, cells transfected with NQO1 significantly enhanced growth inhibition by MMC compared with the mock-transfected and LacZ-transfected cells. We confirmed the cellular expression of the *NQO1* gene product by immunoblot (Fig. 3C). Thus, this screening system can be used to detect changes in chemosensitivity in HT1080 cells. Using this screening system, we examined whether the 19 genes, which were extracted in Tables 2 and 3, altered sensitivity to drug. Notably, the *HSPA1A* gene coding 70-kDa heat shock protein, whose expression was correlated with MMC sensitivity in the breast and liver cancer cell lines, significantly enhanced the MMC sensitivity in HSPA1A-transfected HT1080 cells (Fig. 3B). Similarly, the *JUN* gene encoding c-JUN, whose expression was correlated with MMC sensitivity, also enhanced the MMC sensitivity in JUN-transfected HT1080 cells (Fig. 3B). The expression of *myc*-tagged LacZ, 70-kDa heat shock protein, and JUN in the transfected cells was confirmed by immunoblotting with anti-*myc* antibody (Fig. 3C). Transfection with 17 other genes did not alter the MMC sensitivity. For example, transfection with the *IL-18* gene did not affect MMC sensitivity (Fig. 3B).

Discussion

The assessment system for determining pharmacologic properties of chemicals by a panel of cancer cell lines was first developed in the National Cancer Institute (33–35). We established a similar assessment system (JFCR-39;

Table 3. Genes related to MMC sensitivity in breast, liver, and stomach cancer cell lines

Rank	Gene	Genbank ID	r	P
A. Breast cancer				
Sensitive				
1	*INHBB*	M31682	0.972	0.000
2	*NK4*	M59807	0.838	0.018
3	*HSPA1A*	M11717	0.751	0.050
4	*LOC54557*	AF075050	0.735	0.024
5	*CD47*	Y00815	0.717	0.045
Resistant				
1	*RPN2*	Y00282	−0.882	0.009
2	*ATP5O*	X83218	−0.842	0.017
3	*CAST*	D50827	−0.815	0.025
4	*HPCA*	D16593	−0.776	0.024
5	*ZNF9*	M28372	−0.774	0.024
6	*A2LP*	U70671	−0.772	0.042
7	*IL-18*	D49950	−0.747	0.033
8	*NRGN*	Y09689	−0.727	0.041
B. Liver cancer				
Sensitive				
1	*EB1*	U24166	0.872	0.002
2	*JUN*	J04111	0.813	0.008
3	*EIF3S8*	U46025	0.772	0.015
4	*CTSD*	M11233	0.753	0.012
5	*SCYA5*	M21121	0.741	0.022
6	*PHB*	S85655	0.739	0.023
7	*HSPA1A*	M11717	0.729	0.026
8	*SPP1*	X13694	0.723	0.018
9	*TAB7*	X93499	0.712	0.021
10	*ACTN1*	X15804	0.692	0.039
11	*RXRB*	M84820	0.678	0.045
12	*PSME2*	D45248	0.673	0.047
13	*HLA-C*	M11886	0.647	0.043
14	*RPL19*	X63527	0.643	0.033
Resistant				
1	*MAPK6*	X80692	−0.862	0.003
2	*GCSH*	M69175	−0.793	0.006
3	*G22P1*	M32865	−0.727	0.017
4	*USP11*	U44839	−0.725	0.027
5	*ACTB*	X00351	−0.715	0.020
6	*YWHAZ*	M86400	−0.706	0.022
7	*IL-10*	M57627	−0.694	0.018
8	*RFC4*	M87339	−0.677	0.016
9	*CRLF1*	AF059293	−0.644	0.033
10	*RPS6*	M20020	−0.619	0.042
11	*EMX1*	X68879	−0.618	0.043
12	*TK2*	U77088	−0.607	0.047
C. Stomach cancer				
Sensitive				
1	*TEAD4*	U63824	0.803	0.001
2	*NR2C2*	U10990	0.713	0.001
3	*CSF1*	M37435	0.711	0.004
4	*RAB28*	X94703	0.695	0.008
5	*CBR3*	Ab004854	0.683	0.007
6	*NFYC*	Z74792	0.639	0.019
7	*PGF*	X54936	0.627	0.022

NOTE: Column 2 shows the name of the gene according to HUGO database. Column 4 shows Pearson correlation coefficient between chemosensitivity to drugs and gene expression. "Sensitive" indicates candidate genes sensitive to each drug. "Resistant" indicates genes resistant to each drug.

Table 3. Genes related to MMC sensitivity in breast, liver, and stomach cancer cell lines (Cont'd)

Rank	Gene	Genbank ID	r	P
8	*ERG*	M21535	0.620	0.005
9	*MLLT1*	L04285	0.613	0.015
10	*FOS*	K00650	0.599	0.014
11	*TNFAIP3*	M59465	0.584	0.011
12	*CNR2*	X74328	0.581	0.009
13	*DRPLA*	D31840	0.577	0.024
14	*PSMB5*	D29011	0.572	0.026
15	*SLC6A8*	L31409	0.570	0.017
16	*SERPINB10*	U35459	0.570	0.013
17	*VAT1*	U18009	0.570	0.009
18	*TJP1*	L14837	0.562	0.029
19	*PELP1*	U88153	0.545	0.035
20	*C1QBP*	L04636	0.545	0.024
21	*CDK10*	L33264	0.543	0.045
22	*SERPINA6*	J02943	0.542	0.025
23	*ACTB*	X00351	0.538	0.021
24	*SFRP4*	AF026692	0.538	0.018
25	*EMX1*	X68879	0.535	0.018
26	*ACTB*	X00351	0.529	0.024
27	*RPS9*	U14971	0.528	0.043
28	*AMD1*	M21154	0.522	0.038
29	*RPL26*	X69392	0.522	0.038
30	*HNRPF*	L28010	0.520	0.047
31	*PTMS*	M24398	0.502	0.040
32	*STK12*	AF008552	0.498	0.050
33	*NR2F6*	X12794	0.491	0.046
34	*GBE1*	L07956	0.470	0.049
Resistant				
1	*PSMD8*	D38047	−0.747	0.002
2	*LAMP2*	J04183	−0.677	0.002
3	*CTSD*	M11233	−0.651	0.006
4	*ADORA2B*	M97759	−0.645	0.005
5	*ANXA4*	M19383	−0.639	0.008
6	*PTPRK*	Z70660	−0.638	0.003
7	*RAD23A*	D21235	−0.622	0.010
8	*SDHA*	D30648	−0.613	0.015
9	*PET112L*	AF026851	−0.598	0.024
10	*DAD1*	D15057	−0.593	0.025
11	*HSPB1*	X54079	−0.588	0.013
12	*PSMA6*	X61972	−0.586	0.036
13	*KDELR1*	X55885	−0.584	0.028
14	*B2M*	AB021288	−0.581	0.023
15	*M6PR*	M16985	−0.579	0.038
16	*GCLC*	M90656	−0.576	0.015
17	*SPTBN1*	M96803	−0.557	0.038
18	*PACE*	X17094	−0.547	0.019
19	*RPL24*	M94314	−0.539	0.017
20	*SPINT2*	U78095	−0.538	0.039
21	*STX4A*	U07158	−0.534	0.027
22	*SIAT8B*	U33551	−0.532	0.028
23	*CTSK*	U13665	−0.529	0.029
24	*DCI*	L24774	−0.525	0.044
25	*MEL*	X56741	−0.525	0.045
26	*PITPNB*	D30037	−0.523	0.038
27	*YY1*	M76541	−0.512	0.043
28	*RAB1*	M28209	−0.495	0.037
29	*UBE2L6*	AF031141	−0.492	0.045
30	*PSMB7*	D38048	−0.484	0.049

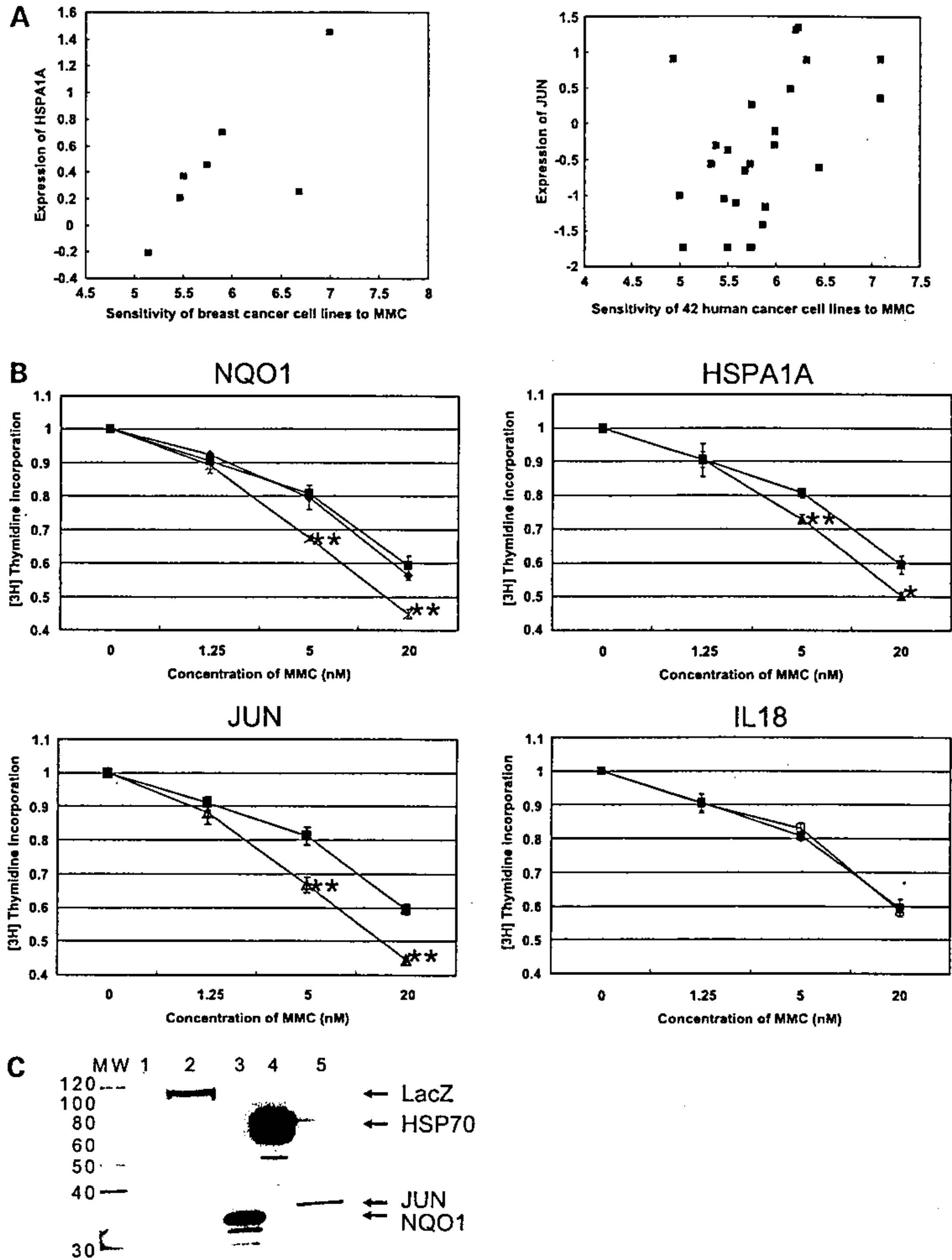

Figure 3. Relationships between MMC sensitivity and expression of HSPA1A in breast cancer cell lines (**A**, *left*) or JUN in 42 cell lines (**A**, *right*). Each symbol indicates one cell line. *X axis*, MMC sensitivity; *Y axis*, expression of HSPA1A or JUN. Pearson correlation coefficients between MMC sensitivity and expression of HSPA1A and JUN were 0.75 (P = 0.05) and 0.473 (P = 0.015), respectively. **B**, growth inhibition curves by MMC in mock (■), LacZ (♦), NQO1 (×), HSPA1A (▲), JUN (△), or IL-18 (□) transfected HT1080 cells. This growth inhibition by MMC was enhanced in HT1080 cells transfected with NQO1, HSPA1A, and JUN. *, $P < 0.002$; **, $P < 0.0001$, *t* test against mock-transfected cells. **C**, expressions of genes were certified by immunoblotting with anti-*myc* antibody: *myc*-tagged LacZ (*lane 2*), NQO1 (*lane 3*), 70-kDa heat shock protein (*HSP70*; *lane 4*), and JUN (*lane 5*).

ref. 32) and showed that drugs with similar modes of actions were classified into the same cluster by hierarchical clustering (19). In this study, we constructed a new panel of 45 human cancer cell lines (JFCR-45), comprising cancer cell lines derived from tumors from three different organ types: breast, liver, and stomach. In particular, the inclusion of cell lines derived from gastric and hepatic cancers is a major point of novelty. JFCR-45 can be used for analyzing both organ-specific differences in chemosensitivity and intraorgan heterogeneity of chemosensitivity. We examined 53 anticancer drugs for their activity against JFCR-45 and observed differential activity across the whole panel as well as within a single organ type (e.g., breast, liver, or stomach). Furthermore, as shown in Fig. 1, using JFCR-45, drugs with a similar mode of action (such as a tubulin binder or topo I inhibitor) were classified into the same cluster, which were the same as the clusters established for NCI-60 (35) and JFCR-39 (19). These results suggest that the cell line panel-based assessment system is generally effective for classifying anticancer drugs with the same modes of action into the same set of clusters.

In this study, we investigated the gene expression profiles of 42 cell lines of JFCR-45 using cDNA array consisting of 3,537 genes. Hierarchical clustering analysis of these gene expression profiles classified organ-specific cell lines mostly into the same cluster, suggesting that these cell lines maintained the genetic characteristics of the parent organ as far as the gene expression profiles were concerned.

We did a Pearson correlation analysis of the gene expression database and the drug sensitivity database. Consequently, many genes whose expressions were correlated with respect to the sensitivity of each drug were identified. For example, DNA alkylating agents and nucleic acid–related genes, including *SF1* encoding ZFM1, *c-JUN* oncogene, and *SFRS9* were extracted as the genes sensitive to MMC. The genes that were sensitive to paclitaxel included tubulin binder and cytoskeleton-related genes, such as *VIL2* encoding ezrin and *ACTB* encoding β-actin. These results suggest that the extracted genes are the predictive markers of drug efficacy. We further applied Pearson correlation analysis to each type (i.e., breast, liver, or stomach cancer) of cell lines. There were two advantages in this type of analysis: one is that we could compare the cell lines having the same organ background and another is that organ-specific genes, which worked as the sensitive or resistant factors, could be extracted. For example, for MMC, several genes (such as *INHBB*, *NK4*, and *HSPA1A*) were newly extracted as candidate genes sensitive to MMC from the breast cancer cell lines. Surprisingly, compared with the breast and liver cancer cells, many new candidate genes were extracted from the stomach cancer cell lines. These extracted genes were considered as the candidates for organ-specific predictive markers of drug efficacy.

We hypothesized that some of the candidate sensitivity genes described above might causally affect the chemosensitivity of cancer cell lines. To validate this possibility, we selected 19 genes, including *HSPA1A*, *JUN*, and *IL-18*, and examined whether the expression of these candidate genes would affect the cellular sensitivity to anticancer drugs. Overexpression of 2 of the 19 genes, *HSPA1A* encoding 70-kDa heat shock protein and *JUN* encoding c-JUN, indeed enhanced cellular sensitivity to MMC in HT1080 cells (Fig. 3), suggesting that they function to mediate MMC sensitivity. This was an unexpected finding, because a direct relationship between these two genes and MMC sensitivity has not been reported previously, although a relationship between heat shock protein and cancer has been suggested previously (36, 37). How these two genes potentiate MMC sensitivity remains to be clarified. In this validation, we used the HT1080 cell line instead of those in JFCR-45 because of its high transfection efficiency. As the alteration of chemosensitivity following the overexpression of any particular gene may depend highly on the genotypic/phenotypic background of the transfected HT1080 cells, further validation using cell lines within JFCR-45 will be required. In addition to the overexpression experiments, validation by silencing chemosensitivity-related genes using small interfering RNA will be required.

Pioneering attempts to discover new leads and targets and to investigate new aspects of the molecular pharmacology of anticancer drugs by mining the NCI-60 database have been done (31, 33-35). Recently, Szakacs et al. (38) have identified interesting compounds whose activity is potentiated by the MDR1 multidrug transporter. Our previous studies using JFCR-39 (19, 20, 31) and the present study using JFCR-45 also indicate that a comprehensive analysis of chemosensitivity and gene expression data followed by experimental validation leads to the identification of genes that determine drug sensitivity.

In conclusion, we established a sensitivity database for JFCR-45, which focused on organ origin, to 53 anticancer drugs. Using JFCR-45, anticancer drugs were classified according to their modes of action. Moreover, we established a database of the gene expression profiles in 42 cell lines of JFCR-45. Using these two databases, we have identified several genes that may predict chemosensitivity of cancer. Among these candidate genes, we identified two genes, *HSPA1A* and *JUN*, which determined sensitivity to MMC. Thus, this approach is useful not only to discover predictive markers for the efficacy of anticancer drugs but also to discover genes that determine chemosensitivity.

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Thyroid hormone-like and estrogenic activity of hydroxylated PCBs in cell culture

Shigeyuki Kitamura[a,*], Norimasa Jinno[a], Tomoharu Suzuki[a], Kazumi Sugihara[a], Shigeru Ohta[a], Hiroaki Kuroki[b], Nariaki Fujimoto[c]

[a] *Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan*
[b] *Daiichi College of Pharmaceutical Sciences, Tamagawa 22-1, Minami-ku, Fukuoka 815-8511, Japan*
[c] *Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan*

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Abstract

The thyroid hormone-disrupting activity of hydroxylated PCBs was examined. 4-Hydroxy-2,2 ,3,4 ,5,5 -hexachlorobiphenyl (4-OH-2,2 ,3,4 ,5,5 -HxCB), 4-hydroxy-3,3 ,4 ,5-tetrachlorobiphenyl (4-OH-3,3 ,4 ,5-TCB) and 4,4 -dihydroxy-3,3 ,5,5 -tetrachlorobiphenyl (4,4 -diOH-3,3 ,5,5 -TCB), which have been detected as metabolites of PCBs in animals and humans, and six other 4-hydroxylated PCBs markedly inhibited the binding of triiodothyronine (1×10^{-10} M) to thyroid hormone receptor (TR) in the concentration range of 1×10^{-6} to 1×10^{-4} M. However, 4-hydroxy-2 ,4 ,6 -trichlorobiphenyl (4-OH-2 ,4 ,6 -TCB), 3-hydroxy-2,2 ,5,5 -tetrachlorobiphenyl, 4-hydroxy-2,2 ,5,5 -tetrachlorobiphenyl, 4-hydroxy-2,3,3 ,4 -tetrachlorobiphenyl, 2,3 ,5,5 -tetrachlorobiphenyl and 2,3 ,4 ,5,5 -pentachlorodiphenyl did not show affinity for TR. The thyroid hormonal activity of PCBs was also examined using rat pituitary cell line GH3 cells, which grow and release growth hormone in a thyroid hormone-dependent manner. 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4,4 -diOH-3,3 ,5,5 -TCB and 4-OH-3,3 ,4 ,5-TCB enhanced the proliferation of GH3 cells and stimulated their production of growth hormone in the concentration range of 1×10^{-7} to 1×10^{-4} M, while PCBs which had no affinity for thyroid hormone receptor were inactive. In contrast, only 4-OH-2 ,4 ,6 -TCB exhibited a significant estrogenic activity using estrogen-responsive reporter assay in MCF-7 cells. However, the 3,5-dichloro substitution of 4-hydroxylated PCBs markedly decreased the estrogenic activity. These results suggest that, at least for the 17 PCB congeners and hydroxylated metabolites tested, a 4-hydroxyl group in PCBs is essential for thyroid hormonal and estrogenic activities, and that 3,5-dichloro substitution favors thyroid hormonal activity, but not estrogenic activity.
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Keywords: Hydroxylated PCB; Thyroid hormonal activity; Thyroid hormone receptor; Estrogenic activity; Rat pituitary tumor cell line GH3; Human breast cancer cell-line MCF-7

1. Introduction

Polychlorinated biphenyls (PCBs) have been widely used throughout the world as industrial chemicals

* Corresponding author. Tel.: +81 82 257 5328; fax: +81 82 257 5329.
E-mail address: skitamu@hiroshima-u.ac.jp (S. Kitamura).

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for heat transfer and electrical insulation. However, they have contaminated almost every component of the global ecosystem, including wildlife and human adipose tissue, breast milk and serum, due to their lipophilic character (Safe, 1990, 1994). They have been reported to have a variety of toxic effects, including immunotoxicity, neurotoxicity, developmental toxicity, hepatotoxic effects, reproductive toxicity and carcinogenesis (Safe, 1984, 1990, 1994; Peterson et al., 1993; Seegal, 1996). They also induce biochemical responses, including induction of phases 1 and 2 drug-metabolizing enzymes (Safe, 1994; Byrne et al., 1987). Many of the PCB-induced toxic responses resemble those observed for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which acts through the aryl hydrocarbon receptor (AhR) signaling pathway (Whitlock, 1993). These results are consistent with the identification of several PCB congeners, which bind to AhR and exhibit AhR agonistic actions (Safe, 1994). In recent years it has become evident that exposure to PCBs can also lead to thyroid hormone disturbances in wildlife and humans (Brouwer et al., 1998; Porterfield and Hendry, 1998). Decreased levels of circulating plasma thyroxin (T4) following PCB exposure have been shown in laboratory animals (Brouwer et al., 1998; Barter and Klaassen, 1994).

Many man-made chemicals, which have been widely released into the environment, are able to mimic or antagonize the biological activity of hormones and to disrupt the endocrine systems of humans and other animals (Colborn, 1995; Andersen et al., 1999). These chemicals, which include PCBs, are known as endocrine-disrupting chemicals. Interactions of estrogenic and anti-androgenic compounds with the respective hormone receptors have been demonstrated to account, among others, for endocrine-disrupting actions. PCBs are metabolized in vivo to hydroxylated PCBs, and these metabolites were identified in human serum, wildlife samples, and laboratory animals treated with PCBs (Jansson et al., 1975; Bergman et al., 1994; Morse et al., 1996; Darnerud et al., 1996; Sandau et al., 2000, 2002). Korach et al. (1988) reported that some hydroxy-PCBs such as 4,4 -dihydroxy-3,3 ,5,5 -tetrachlorobiphenyl competitively bind to the estrogen receptor (ER) and exhibit estrogenic activity in the mouse uterus. Other reports also show that hydroxylated PCBs are estrogenic or anti-estrogenic with respect to binding for ER (Moore et al., 1997; Ramamoorthy et al., 1997). Connor et al. (1997) demonstrated complex structure–estrogenicity/anti-estrogenicity relationships for hydroxylated PCBs.

It has also been reported that hydroxylated PCBs can disrupt thyroid hormone status. Hydroxylated PCBs showed high binding affinity for the serum thyroid hormone binding protein transthyretin, thereby displacing the natural ligand, T4 (Lans et al., 1993, 1994; Cheek et al., 1999). In vivo toxicity of hydroxylated PCBs on thyroid hormone homeostasis was also suggested on the basis of their high binding affinity to transthyretin (Darnerud et al., 1996; Meerts et al., 2002). However, the exact mechanisms of interference with thyroid hormonal action are not fully understood. Recently, we reported that tetrabromobisphenol A, a flame retardant, exhibits thyroid hormonal activity through its affinity to the thyroid hormone receptor (TR) (Kitamura et al., 2002). In the present report, we deal with the thyroid hormonal and anti-thyroid hormonal activities of fourteen hydroxylated PCBs and three non-hydroxylated PCBs as examined by means of binding assay with TR, as well as thyroid hormone-dependent growth assay and assay of production of growth hormone (GH) in pituitary cell line GH3 cells. The results were compared with the estrogenic activity observed in ERE-luciferase reporter assay. As substrates, we used 4-hydroxy-2,2 ,3,4 ,5,5 -hexachlorodiphenyl (4-OH-2 2 ,3,4 ,5,5 -HxCB), 4-hydroxy-3,3 ,4 ,5-tetrachlorobiphenyl (4-OH-3,3 ,4 ,5-TCB), 4,4 -dihydroxy-3,3 ,5, 5 -tetrachlorobiphenyl (4,4 -diOH-3,3 ,5,5 -TCB), 3-hydroxy-2,2 ,5,5 -tetrachlorobiphenyl (3-OH-2,2 ,5, 5 -TCB) and 4-hydroxy-2,2 ,5,5 -tetrachlorobiphenyl (4-OH-2,2 ,5,5 -TCB), which are metabolites of PCBs in animals and humans, as well as other hydroxylated PCBs (see Fig. 1).

2. Materials and methods

2.1. Chemicals

L-3,5,3 -Triiodothyronine (T3; 98%) and 17 -estradiol (E2; >98%) were obtained from Sigma Chemical Co. (St. Louis, MO). ^{125}I-T3 (3,5,3 -^{125}I, radiochemical purity >95%, 28.8 TBq/mmol) was purchased from NEN Life Science Products, Boston, MA. 4,4 -DiOH-3,3 ,5,5 -TCB, 3-OH-2,2 ,5,5 -TCB, 2-hydroxy-3,3 ,

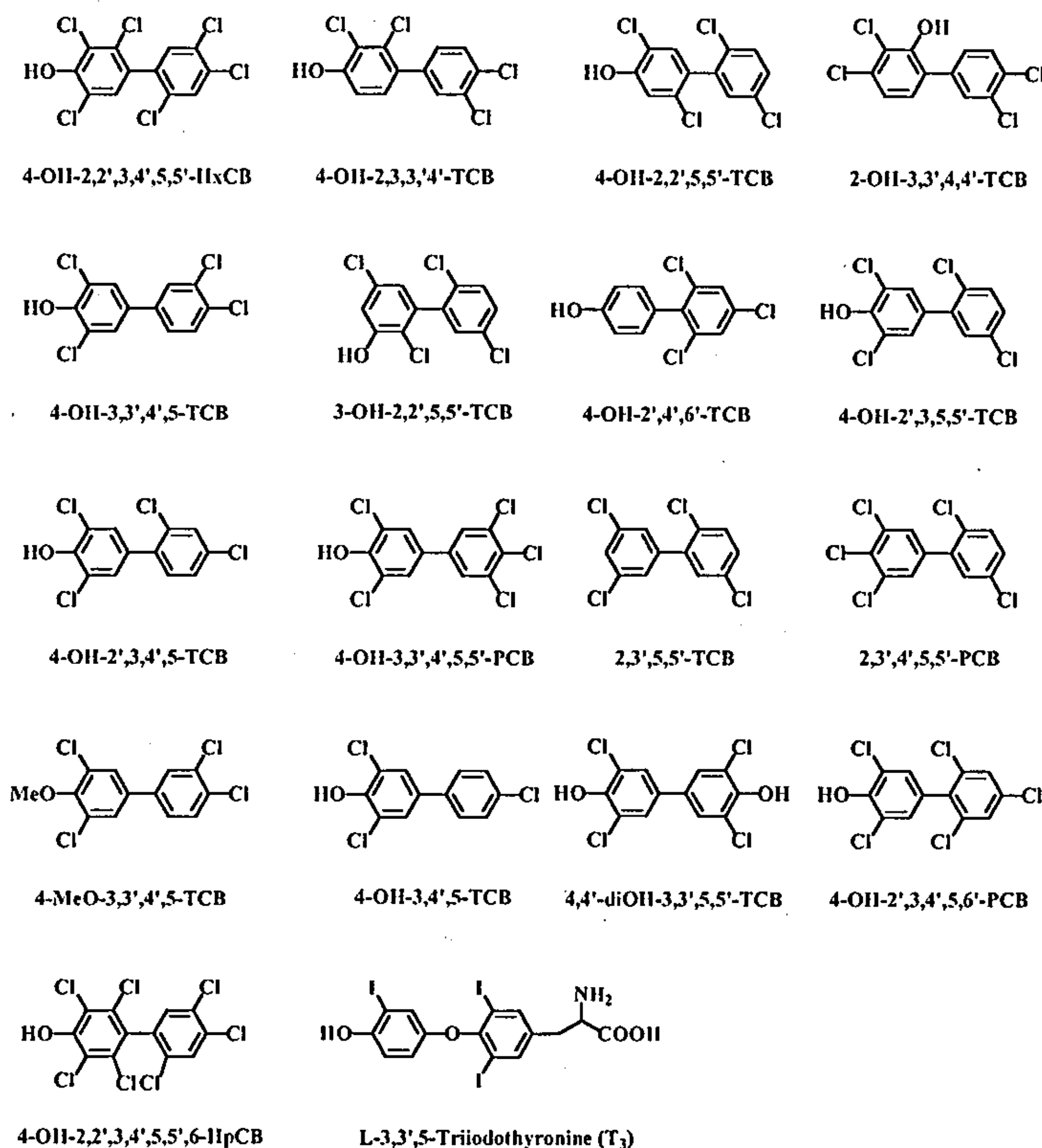

Fig. 1. Structures of PCBs and hydroxylated PCBs.

4 ,4 -tetrachlorobiphenyl (2-OH-3,3 ,4,4 -TCB), 4-methoxy-3,3 ,4 ,5-pentachlorobiphenyl (4-MeO-3,3 , 4 ,5-TCB) and eleven 4-hydroxylated PCBs, 4-OH-2, 2 ,3,4 ,5,5 -HCB, 4-OH-3,3 ,4 ,5-TCB, 4-OH-2,2 , 5,5 -TCB, 4-hydroxy-2 ,4 ,6 -trichlorobiphenyl (4-OH-2 ,4 ,6 -TCB), 4-hydroxy-3,4 ,5-trichlorobiphenyl (4-OH-3,4 ,5-TCB), 4-hydroxy-3,3 ,4 ,5,5 -pentachlorobiphenyl (4-OH-3,3 ,4 ,5,5 -PCB), 4-hydroxy-2 , 3, 5,5 -tetrachlorobiphenyl (4-OH-2 ,3,5,5 -TCB), 4-hydroxy-2,3 ,4 ,5,6 -pentachlorobiphenyl (4-OH-2 ,3,4 ,5,6 -PCB), 4-hydroxy-2,3,3 ,4 -tetrachlorobiphenyl (4-OH-2,3,3 ,4 -TCB), 4-hydroxy-2 ,3,4 ,5-tetrachlorobiphenyl (4-OH-2 ,3,4 ,5-TCB) and 4-hydroxy-2,2 ,3,4 ,5,5 ,6-heptachlorobiphenyl (4-OH-2,2 ,3,4 ,5,5 ,6-HpCB) were synthesized by the previously reported method (Bergman et al., 1994). 2,3 ,5,5 -Tetrachlorobiphenyl (2,3 ,5,5 -TCB) and 2,3 ,4 ,5,5 -pentachlorobiphenyl

(2,3 ,4 ,5,5 -PCB) were obtained from Accu Standard (New Haven, CT).

2.2. Cell culture

A rat pituitary cell line, GH3 and a human breast cancer cell line, MCF-7, were obtained from the Health Science Research Resources Bank (Osaka, Japan). The other rat pituitary cell line, MtT/E-2, was established in our laboratory (Fujimoto et al., 1999). GH3 and MtT/E-2 were maintained in DME/F12 mixed medium (Sigma Chemical Co.) containing penicillin and streptomycin with 15% horse serum (HS, Life Technologies) and 2.5% fetal bovine serum (FBS). Before cell growth assay and GH production assay, GH3 cells were maintained for 2–3 days in phenol-red free DEM/F12 (Sigma Chemical Co.) containing the same antibiotics along with dextran–charcoal treated HS and FBS. In the case of an estrogen-responsive human breast cancer cell-line MCF-7, cells were maintained in MEM (Sigma Chemical Co.) containing penicillin and streptomycin with 5% fetal bovine serum (Life Technologies, Rockville, MD).

2.3. Competitive binding assay to thyroid hormone receptor

Nuclear extracts of MtT/E-2 were used for the assay, since this cell line expresses high amounts of thyroid hormone receptor. The cells were homogenized in 0.32 M sucrose solution containing 3 mM $MgCl_2$ and 1 mM dithiothreitol and centrifuged at $700 \times g$ for 10 min. The pellets were resuspended in 2.4 M sucrose with $MgCl_2$ and centrifuged at $53,000 \times g$ for 45 min. The resulting nuclear pellets were resuspended in TMDS buffer (2 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.32 M sucrose, pH 7.4). Various concentrations of test chemicals and 3 nM ^{125}I-T3 were incubated in 0.2 ml of the nuclear suspension at 37 C for 40 min. After incubation, 0.25 ml of 2% Triton X-100 was added to terminate the reaction, and the mixture was centrifuged at $1000 \times g$ for 10 min. The pellets were washed twice with 1 ml of TMDS buffer and the supernatant was removed. Radioactivity of the pellets was counted with a gamma counter (Amersham Pharmacia Biotech, Uppsala, Sweden). IC_{50} values were calculated by fitting data to the logistic equation.

2.4. GH production assay in GH3 cells

The assay was performed as reported previously (Kitamura et al., 2002). Briefly, GH3 cells were seeded in 24-well plates at 1×10^4 cells/well and chemicals dissolved in 10 1 of ethanol were added the next day. Two days later, growth hormone in the culture medium was measured by radioimmunoassay with NIADDK reagents following the recommended protocol. The amount of growth hormone release at the maximum response (T3, 1×10^{-8} M) was 96.0 9.7 ng/10^5 cells/day.

2.5. Thyroid hormone-dependent growth assay in GH3 cells

The cells were seeded in 24-well plates at 1×10^4 cells/well, and chemicals were added on the following day. One week later, cell growth was measured with a modified MTT assay kit, which employs a newly developed tetrazolium salt, WST-1 (Dojindo Chemicals, Kumamoto, Japan). The details were previously reported (Kitamura et al., 2002).

2.6. Assay of estrogenic activity of hydroxylated PCBs

ERE-luciferase reporter assay using MCF-7 cells was performed according to the previously reported method (Kitamura et al., 2003). Briefly, transient transfections in MCF-7 cells were performed with Transfast™ (Promega Co., Madison, WI), using the manufacturer's protocol. Transfections were done in 12-well plates at 1×10^5 cells/well with 1.9 g of p(ERE)$_3$-SV40-luc and 0.1 g of pRL/CMV (Promega Co.) as an internal standard. Twenty-four hours after addition of the sample (final concentration, 10^{-4} to 10^{-9} M), the assay was performed with a Dual Luciferase assay kit™ (Promega Co.).

2.7. Statistics

Results are expressed as the mean S.D. Multiple comparison was made by ANOVA followed by Scheffe's test.

3. Results

3.1. Competitive binding assay for thyroid hormone-like compounds

The inhibitory effects of PCBs on binding of T3 to TR were examined using nuclear fraction of MtT/E-2 cells. T3 competitively inhibited the binding of ^{125}I-T3 (1×10^{-10} M) to TR in the range of 1×10^{-9} to 1×10^{-8} M. 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4-OH-3,3 ,4 ,5-TCB and 4,4 -diOH-3,3 ,5,5 -TCB, which have been detected as metabolites of PCBs in humans and other animals, also markedly inhibited the binding of ^{125}I-T3 to the receptor in the concentration range of 1×10^{-5} to 1×10^{-4} M (Fig. 2A and B). Furthermore, some 4-hydroxylated PCBs, 4-OH-2 ,3,5,5 -TCB, 4-OH-3,3 ,4 ,5,5 -PCB, 4-OH-2,2 ,3,4 ,5,5 ,6-HpCB, 4-OH-3,4 ,5-TCB, 4-OH-2 ,3,4 ,5,6 -PCB and 4-OH-2 ,3,4 ,5-TCB, examined in this study showed binding affinity to TR (Fig. 2A–C). 2,3 ,5,5 -TCB, 2,3 ,4 ,5,5 -PCB, 4-OH-2 ,4 ,6 -TCB, 2-OH-3,3 ,4,4 -TCB, 3-OH-2,2 ,5,5 -TCB, 4-OH-2,3,3 ,4 -TCB and 4-OH-2,2 ,5,5 -TCB showed little or no affinity (data not shown). As evidenced by the IC_{50} values for TR binding of PCBs (Table 1), elevated binding affinity was observed for 4-hydroxylated PCBs with chlorine substitution at both the 3- and 5-positions of the phenyl group, including 4,4 -diOH-3,3 ,5,5 -TCB. Among these compounds, 4-OH-2,2 ,3,4 ,5,5 ,6-HpCB exhibited the greatest activity, followed by 4-OH-2,3,4 ,5,6 -PCB, 4-OH-3,3 ,4,5,5 -PCB, 4-OH-3,3 ,4 ,5-TCB. 4-Hydroxy-PCBs chlorinated at the 3- or 5-position, but not both, showed little activity. PCBs with a hydroxyl group at the 3- or 2-position of the phenyl ring, and 4-methoxy-3,3 ,4 ,5-TCB also showed little affinity.

Table 1
IC_{50} values of PCBs for binding to thyroid hormone receptor

Compound	IC_{50} (M)
T3	0.0033
4-OH-2,2 ,3,4 ,5,5 ,6-HpCB	4.6
4-OH-2 ,3,4 ,5,6 -PCB	9.1
4-OH-3,3 ,4 ,5,5 -PCB	9.3
4-OH-3,3 ,4 ,5-TCB	9.9
4-OH-2 ,3,4 ,5-TCB	16
4-OH-3,4 ,5-TCB	22
4-OH-2,2 ,3,4 ,5,5 -HxCB	34
4,4 -diOH-3,3 ,5,5 -TCB	42
4-OH-2 ,3,5,5 -TCB	53
2,3 ,5,5 -TCB	>1000
2,3 ,4 ,5,5 -PCB	>1000
4-OH-2,2 ,5,5 -TCB	>1000
3-OH-2,2 ,5,5 -TCB	>1000
4-OH-2,3,3 ,4 -TCB	>1000
2-OH-3,3 ,4,4 -TCB	>1000
4-OH-2 ,4 ,6 -TCB	>1000
4-Methoxy-3,3 ,4 ,5-TCB	>1000

3.2. Thyroid hormonal activity of hydroxylated PCBs evaluated by assay of GH production by GH3 cells

The thyroid hormonal activities of hydroxylated PCBs were examined by measuring the ability of these compounds to induce the production of GH by GH3 cells using 4-hydroxylated PCBs detected as metabolites in humans and other animals. GH release activity was observed with T3 in the range of 1×10^{-10} to 1×10^{-8} M. When GH release from GH3 cells was measured after the addition of 4-OH-2,2 ,3,4 ,5,5 -HxCB and 4-OH-3,3 ,4 ,5-TCB, an increase was observed in the concentration range of 1×10^{-6} to 1×10^{-5} M (Fig. 3A). Positive result was also observed in 4,4 -diOH-3,3 ,5,5 -TCB at 1×10^{-5} M (Fig. 3B). However, 2,3 ,5,5 -TCB, 3-OH-2,2 ,5,5 -TCB and 4-OH-2,2 ,5,5 -TCB showed no significant effect (Fig. 3A and B). The inhibitory effects of PCBs on the hormonal activity of T3 on GH3 cells were examined. These compounds at 1×10^{-5} and 1×10^{-4} M did not show antagonistic action towards GH production induced by 1×10^{-10} and 1×10^{-9} M T3 (data not shown). These results suggest that some hydroxylated PCBs tested in this study act as thyroid hormone agonists, but not antagonists.

3.3. Thyroid hormonal activity of hydroxylated PCBs evaluated by growth assay of GH3 cells

The thyroid hormonal activity of hydroxylated PCBs was also examined by assay of thyroid hormone-dependent growth of GH3 cells. The growth-inducing effect of T3 on the cells was observed over the concentration range of 1×10^{-10} to 1×10^{-8} M. When 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4-OH-3,3 ,4 ,5-TCB and 4,4 -diOH-3,3 ,5,5 -TCB were added to the cells, growth was also stimulated at 1×10^{-6} to 1×10^{-5} M. 4-OH-

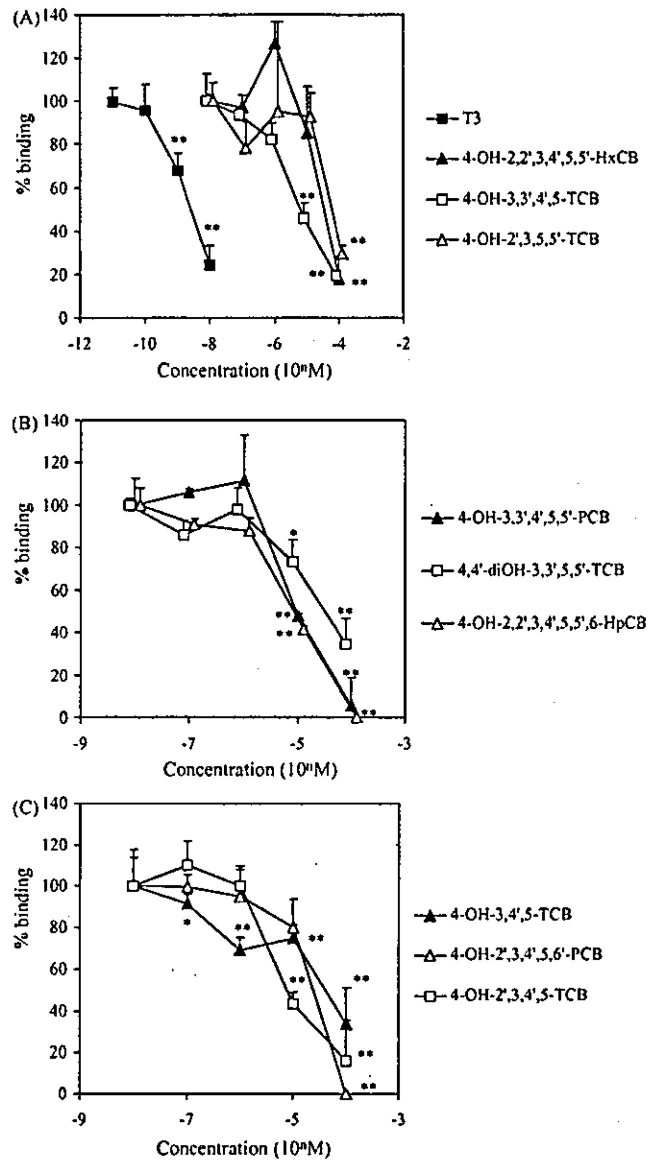

Fig. 2. Binding assay of thyroid hormonal compounds to thyroid hormone receptor. The nine positive hydroxylated PCBs are divided into three groups (A–C) simply for clarity. Each value represents the mean S.D. of four experiments. Activity is expressed relative to the control with no added test compound. T3; L-3,5,3 -triiodothyronine. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

2,2 ,3,4 ,5,5 -HxCB showed the highest activity, followed by 4-OH-3,3 ,4 ,5-TCB and 4,4 -diOH-3,3 ,5,5 -TCB (Fig. 4). However, 2,3 ,5,5 -TCB, 3-OH-2,2 ,5,5 -TCB and 4-OH-2,2 ,5,5 -TCB showed no significant effect. No cytotoxic effect of these positive hydroxylated PCBs towards GH3 cells was observed in this concentration range. These compounds at concentrations of 1×10^{-5} and 1×10^{-4} M did not inhibit the induction of GH3 cell growth by 1×10^{-10} and 1×10^{-9} M T3 (data not shown). These results confirmed that the hydroxylated PCBs examined in this assay, 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4-OH-3,3 ,4 ,5-TCB

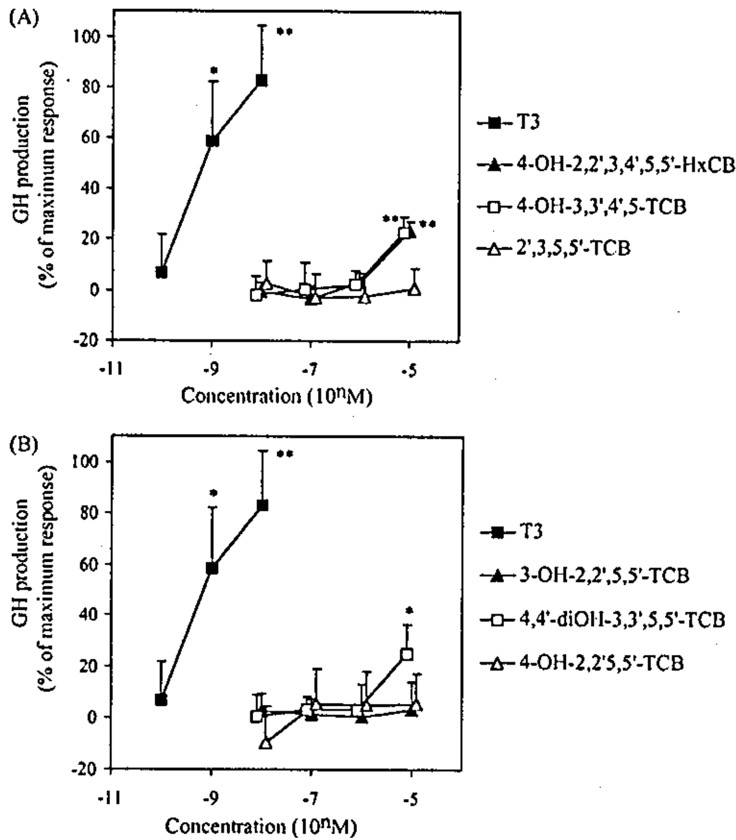

Fig. 3. Growth hormone (GH) release from GH3 cells by hydroxylated PCBs. Three positive compounds and three negative compounds are shown in two panels simply for clarity. Each bar represents the mean S.D. of four experiments. Activity is expressed relative to the control untreated GH3 cells. The amount of growth hormone release at the maximum response (T3, 1×10^{-8} M) was 96.0 9.7 ng/10^5 cells/day. T3; L-3,5,3 -triiodothyronine. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

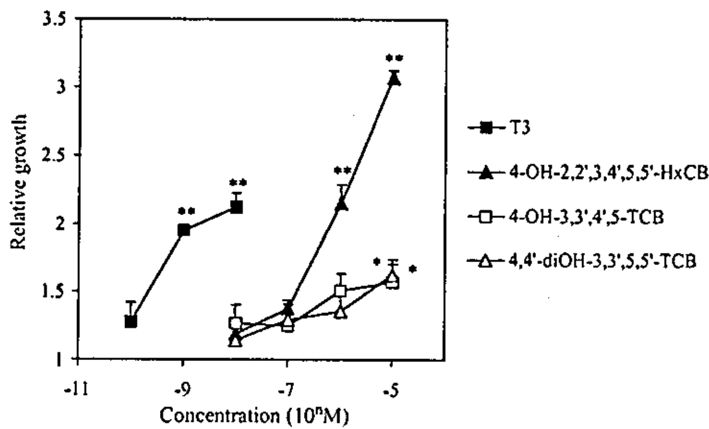

Fig. 4. Growth of GH3 cells by hydroxylated PCBs. Each bar represents the mean S.D. of four experiments. Activity is expressed relative to the control untreated GH3 cells. T3; L-3,5,3 -triiodothyronine. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

and 4,4 -diOH-3,3 ,5,5 -TCB, act as thyroid hormone-disruptors by exhibiting agonistic activity.

3.4. Estrogenic activity of hydroxylated PCBs

The estrogenic activities of hydroxylated PCBs were examined using ERE-luciferase reporter assay in MCF-7 cells. When 4-OH-2 ,4 ,6 -TCB was added to the cells in the concentration range of 1×10^{-7} to 1×10^{-5}M, estrogenic activity was apparent in the estrogen screening assay (Fig. 5A). 4-OH-2,3,3 ,4 -TCB, 2-OH-3,3 ,4,4 -TCB, 4-OH-2,2 ,5,5 -TCB, 4-OH-3,3 ,4 ,5-TCB and 3-OH-2,2 ,5,5 -TCB exhibited low activity in the concentration of 1×10^{-5}M. These hydroxylated PCBs were not cytotoxic to MCF-7 cells at concentrations below 1×10^{-5}M (Fig. 5A and B). However, no estrogenic activity of 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4,4 -diOH-3,3 ,5,5 -TCB, 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4-OH-3,3 ,4 ,5-TCB, 3-OH-2,2 ,5,5 -TCB, 4-OH-2,2 ,5,5 -TCB, 2,3 ,5,5 -TCB or 2,3 ,4 ,5,5 -PCB was observed (data not shown). These experiments indicate that a hydroxyl group of PCBs is essential for estrogenic activity, but 4-hydroxyl PCBs with an *ortho*-chlorine substituent show decreased estrogenic activity, though thyroid hormonal activity is unaffected.

4. Discussion

Hydroxylated PCBs have been detected in wildlife and humans as metabolites of PCBs (Jansson et al., 1975; Bergman et al., 1994; Sandau et al., 2000). Here, we present the first evidence that some hydroxylated PCBs, which have been detected as metabolites of PCBs, exhibit thyroid hormonal activity through interaction with TR. There are at least another three mechanisms through which environmental contaminants interact with the thyroid hormone system. These are: (1) direct toxicity at the thyroid gland, which can lead to decreased synthesis of thyroid hormones (Collins and Capen, 1980), (2) disturbance of thyroid hormone metabolism, such as glucuronidation, sulfation or deiodination (Van Birgelen et al., 1995; Brouwer et al., 1998), and (3) interaction with thyroid hormone transport proteins, leading to a reduced plasma T4 level

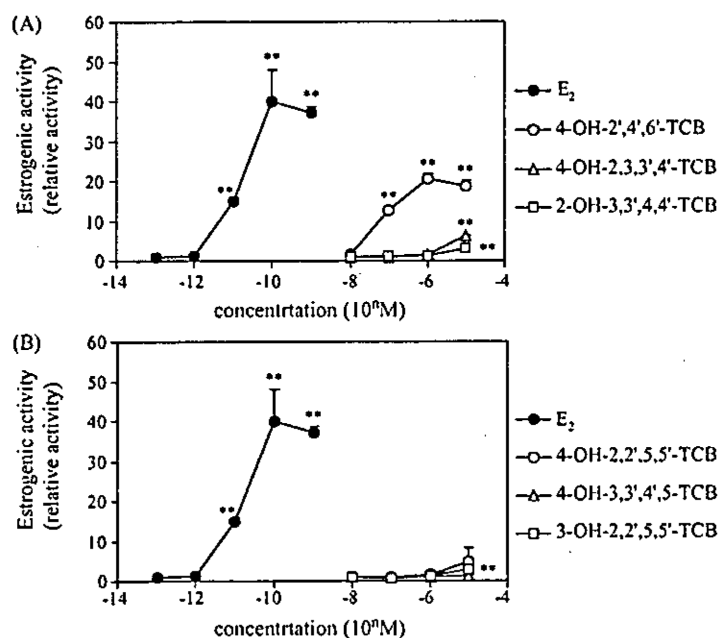

Fig. 5. Estrogenic activity of hydroxylated PCBs using estrogen-reporter assay of MCF-7 cells. Results for 6 hydroxylated PCBs are shown in two panels simply for clarity. Each value represents the mean S.D. of four experiments. Estrogenic activity is expressed as fold change of ERE-luciferase activity over the vehicle control. E2; 17 -estradiol. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

(Lans et al., 1993, 1994; Morse et al., 1996). Various chemicals have been reported to bind to transthyretin, one of the thyroid hormone-binding transport proteins in plasma of vertebrate species. PCBs bind to human transthyretin and thyroid-binding globulin in vitro, competing with thyroid hormone binding (Cheek et al., 1999; Lans et al., 1994; Meerts et al., 2002). In the current study, it was shown that nine hydroxylated PCBs interact with thyroid hormonal receptor. These results suggest that hydroxylated PCBs have the potential to disrupt thyroid hormonal activity in vivo by interaction with TR, besides binding with transthyretin.

We demonstrated that of the molecules tested only hydroxylated PCBs, but not PCBs themselves, have agonistic activity for thyroid hormone using GH3 cells, which respond to thyroid hormone. In other words, hydroxylated PCBs are active metabolites of PCBs from the viewpoint of disrupting thyroid hormonal activity at the cellular level. The levels of these active metabolites in the body would be critical in determining whether these compounds actually show activity in vivo. Sandau et al. (2000, 2002) reported that the amount of total hydroxylated PCBs in normal human populations is of the order of 0.15–0.46 ng/g wet weight of blood, but in highly exposed populations, such as the Inuits, the concentration can reach 11.6 ng/g wet weight of blood at a maximum level. The average values for Inuit men and women are 1.73 and 1.01 ng/g blood, respectively. The concentration of hydroxylated PCBs in humans is thus two or three orders of magnitude less than the concentration shown to cause thyroid hormonal activity in this in vitro study. Therefore, these effects may not occur in the real world of humans and other animals world because of the PCB concentration. High levels of hydroxylated PCBs in marine mammals may be expected (Hoekstra et al., 2003). These compounds may be accumulated in the animal body, especially marine mammals, through the generations and by bioconcentration in the food chain. Hydroxylated PCBs may exhibit thyroid hormonal activity as mixtures of congeners or in combination with other environmental contaminants, such as TCDD. The apparent affinity of these xenobiotics for the thyroid hormone receptor is lower than that of endogenous thyroid hormones. Nevertheless, serum thyroid hormone levels are tightly regulated through the hypothalamus–pituitary thyroid axis in vivo, and this may not be the case for these chemicals, which may act directly on the endocrine organs. In contrast to the present results, it was reported that hydroxylated PCBs disrupt the central nervous system through antagonistic action against thyroid hormones (Iwasaki et al., 2002; Miyazaki et al., 2004). If hydroxylated PCBs are taken up into fetuses, these compounds may induce thyroid hormone-disrupting effects through this action in combination with the agonistic mechanism shown in the present study. Darnerud et al. (1996) also reported that hydroxylated PCBs bind with fetal transthyretin, and this is accompanied with a decrease in fetal plasma thyroxin levels. Further work is necessary to assess the in vivo endocrine-disrupting action of hydroxylated PCBs, taking into account the other thyroid hormone-disrupting actions of these compounds.

In this study, the potency of hydroxylated PCBs to compete for binding with TR was tested, in view of their structural resemblance to the thyroid hormones. Recently, thyroid hormone-disrupting action of some hydroxy-PCBs has been discussed. These hydroxy-PCBs were reported to have binding capability to TR, as well as the serum transport protein transthyretin (Cheek et al., 1999; Connor et al., 1997). Iwasaki et al. (2002) reported that a synthesized hydroxylated PCB, 4-hydroxy-2′,3,3′,4′,5′-pentachlorobiphenyl, acts as an antagonist by suppression of the interaction of TR and a coactivator. In the current study using GH3 cells, we found thyroid hormone agonistic activities of hydroxylated PCBs that have been detected as metabolites in the body. A rat pituitary cell line, GH3, isolated from a rat pituitary tumor has been widely used as a standard pituitary cell model. Cell proliferation, as well as growth hormone secretion, has been shown to depend on thyroid hormones, but only slightly on estrogen (Perrone et al., 1980; Kitagawa et al., 1987). The reason for the difference between the agonistic and antagonistic actions of hydroxy-PCBs may be due to the different responses of the cells used.

We found that PCB congeners substituted with two chlorine atoms adjacent to the hydroxyl group on an aromatic ring show thyroid hormonal activity, though other PCBs do not. It is important to understand the structural requirements for thyroid hormonal activity in xenobiotics. A 4-hydroxyl group and adjacent 3,5-chlorine substituents on the phenyl group seem to be essential structural factors for binding to TR. In this respect, they apparently resem-

ble T4, rather than T3. However, the atomic size of chlorine is much smaller than that of iodine. In view of the affinity of hydroxy-PCBs for TR, 3,5-dichloro substituents may be favorable for binding to TR. We also recognized a similar structural requirement for TR affinity in a halogenated bisphenol A, tetrabromobisphenol A, a flame retardant (Kitamura et al., 2002). Another chlorinated phenyl ring substituted at the 1-position of the phenyl ring bearing the 4-hydroxyl group also seems to be necessary for thyroid hormonal activity, because 4-hydroxy-3,5-dichlorobenzene lacks the activity (data not shown). 2,2 ,6 or 6 -Chloro substitution on aromatic rings did not influence the activity. In other words, there is not a difference in thyroid hormonal activity between coplanar and non-coplanar PCBs. We also tested whether PCBs, including hydroxylated PCBs, exhibit estrogenic activity. We found that 4-OH-2 ,4 ,6 -TCB, a 4-hydroxy-PCB without 3,5-chlorine atoms, is estrogenic by means of an estrogen-responsive reporter test in a human breast cancer cell line MCF-7. However, 4-hydroxy-3,5-dichlorinated PCBs exhibited little estrogenic activity. These results are consistent with the estrogenic activity of hydroxylated PCBs reported elsewhere (Korach et al., 1988; Ramamoorthy et al., 1997). It is interesting that when 4-hydroxy-PCBs have 3,5-dichloro substitution, their estrogenic activity is markedly decreased, whereas their thyroid hormonal activity is not. The results suggest that hydroxylated PCBs exhibit endocrine-disrupting action via effects on at least two different hormonal activities in vivo. In conclusion, the structural requirements of hydroxylated PCBs for thyroid hormonal activity are a 4-hydroxyl group and 3,5-dichloro atoms substituted adjacent to the hydroxyl group. The requirement for estrogenic activity is a 4-hydroxyl group, but 3,5-dichloro substitution of a 4-hydroxy-PCB reduces the estrogenic activity.

Acknowledgments

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Comparative Study of the Endocrine-Disrupting Activity of Bisphenol A and 19 Related Compounds

Shigeyuki Kitamura,*[,1] Tomoharu Suzuki,* Seigo Sanoh,* Ryuki Kohta,* Norimasa Jinno,* Kazumi Sugihara,* Shin'ichi Yoshihara,* Nariaki Fujimoto,† Hiromitsu Watanabe,† and Shigeru Ohta*

*Graduate School of Biomedical Sciences and †Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

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The endocrine-disrupting activities of bisphenol A (BPA) and 19 related compounds were comparatively examined by means of different *in vitro* and *in vivo* reporter assays. BPA and some related compounds exhibited estrogenic activity in human breast cancer cell line MCF-7, but there were remarkable differences in activity. Tetrachlorobisphenol A (TCBPA) showed the highest activity, followed by bisphenol B, BPA, and tetramethylbisphenol A (TMBPA); 2,2-bis(4-hydroxyphenyl)-1-propanol, 1,1-bis(4-hydroxyphenyl)propionic acid and 2,2-diphenylpropane showed little or no activity. Anti-estrogenic activity against 17β-estradiol was observed with TMBPA and tetrabromobisphenol A (TBBPA). TCBPA, TBBPA, and BPA gave positive responses in the *in vivo* uterotrophic assay using ovariectomized mice. In contrast, BPA and some related compounds showed significant inhibitory effects on the androgenic activity of 5α-dihydrotestosterone in mouse fibroblast cell line NIH3T3. TMBPA showed the highest antagonistic activity, followed by bisphenol AF, bisphenol AD, bisphenol B, and BPA. However, TBBPA, TCBPA, and 2,2-diphenylpropane were inactive. TBBPA, TCBPA, TMBPA, and 3,3'-dimethylbisphenol A exhibited significant thyroid hormonal activity towards rat pituitary cell line GH3, which releases growth hormone in a thyroid hormone-dependent manner. However, BPA and other derivatives did not show such activity. The results suggest that the 4-hydroxyl group of the A-phenyl ring and the B-phenyl ring of BPA derivatives are required for these hormonal activities, and substituents at the 3,5-positions of the phenyl rings and the bridging alkyl moiety markedly influence the activities.

Key Words: estrogenic activity; anti-androgenic activity; thyroid hormonal activity; bisphenol A; bisphenol derivative; human breast cancer cell line MCF-7; rat pituitary cell line GH3.

Bisphenol A (2,2-bis-(4-hydroxyphenyl)propane; BPA) is an industrial raw material for polycarbonate and epoxy resins, and contaminates the end products. It can be detected in liquid from canned vegetables and in the saliva of patients treated with dental sealants (Brotons *et al.*, 1995; Hashimoto *et al.*, 2001; Olea *et al.*, 1996). BPA has the ability to bind DNA after metabolic activation (Atkinson and Roy, 1995). BPA also shows estrogenic activity towards cell lines such as estrogen-responsive breast cancer cell line MCF-7 cells, and endocrine-disrupting effects *in vivo* (Ashby and Tinwell, 1998; Ashby *et al.*, 2000; Gaido *et al.*, 1997; Kim *et al.*, 2001; Krishnan *et al.*, 1993; Matthews *et al.*, 2001; Tinwell *et al.*, 2000). Bisphenol B (2,2-bis-(4-hydroxyphenyl)butane; BPB), bisphenol F (4,4'-dihydroxydiphenylmethane; BPF), bisphenol AD (ethylidenebisphenol; BPAD), bisphenol AF (1,3-trifluoro-2,2-bis-(4-hydroxyphenyl)propane; BPAF), tetramethylbisphenol A (2,2-bis-(3,5-dimethyl-4-hydroxyphenyl)propane; TMBPA), 3,3'-dimethylbisphenol A (DMBPA), and bisphenol S (bis-(4-hydroxyphenyl)sulfone; BPS) are also used as materials for polycarbonate resin. Tetrabromobisphenol A (2,2-bis-(3,5-dibromo-4-hydroxyphenyl)propane; TBBPA), a halogenated derivative of BPA, is also widely used throughout the world as a flame retardant in numerous products. TBBPA was developed as a relatively nontoxic flame retardant (Helleday *et al.*, 1999; Sellström and Jansson, 1995; Sjödin *et al.*, 2001; Thomsen *et al.*, 2001; Watanabe *et al.*, 1983). Tetrachlorobisphenol A (2,2-bis-(3,5-dichloro-4-hydroxyphenyl)propane; TCBPA) has been found in the effluent from waste-paper recycling plants (Fukazawa *et al.*, 2001). Kuruto-Niwa *et al.* (2002) reported that estrogenic polychlorinated BPAs were not easily biodegraded. However, the endocrine-disrupting activity of these halogenated BPA has not been reported in detail.

Endocrine-active chemicals arise from many different sources, including pesticides, industrial chemicals, pharmaceuticals, and phytochemicals. These chemicals are widely distributed in the environment, and are able to mimic or antagonize the biological functions of natural hormones. Chlorinated insecticides, such as kepone, *o,p'*-DDT, dieldrin and methoxychlor, and compounds used in the plastics and detergent industries, such as alkylphenols and BPA, are known to have estrogenic activity (Andersen *et al.*, 1999). *p,p'*-DDE, a metabolite of *p,p'*-DDT, vinclozolin, an antifungal agent, and chlornitrofen, fenitrothion and fenthion, insecticides, have anti-androgenic

[1] To whom correspondence should be addressed at Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan. Fax: +81-82-257-5329. E-mail: skitamu@hiroshima-u.ac.jp.

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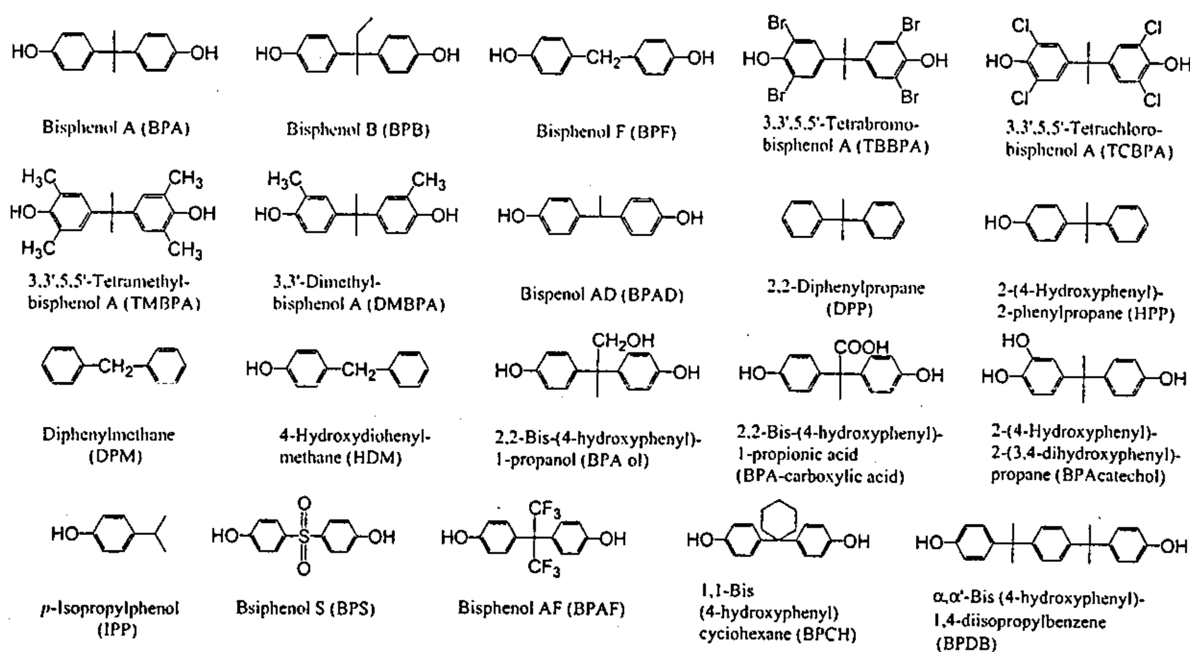

FIG. 1. Structures of bisphenol A and related compounds tested in this study.

activity (Gray *et al.*, 1999; Kelce *et al.*, 1995; Kitamura *et al.*, 2003a; Kojima *et al.*, 2003; Tamura *et al.*, 2001). Some hydroxy-PCBs such as 4,4'-dihydroxy-3,3',5,5'-tetrachlorobiphenyl are reported to show anti-thyroid hormonal activity in addition to estrogenic activity (Cheek *et al.*, 1999; Connor *et al.*, 1997; Korach *et al.*, 1988; Lans *et al.*, 1994). Interactions of estrogenic and anti-androgenic compounds with the respective hormone receptors have been demonstrated to account for most of the endocrine-disrupting actions, and these chemicals can alter reproductive development in mammals. It is also necessary to consider the activity of the metabolites of these chemicals. In the metabolism of BPA, the 3-hydroxyl metabolite (BPA catechol) was formed by human and rat liver microsomes and exhibited estrogenic activity (Elsby *et al.*, 2001). The glucuronide metabolite proved to have no estrogenic activity (Matthews *et al.*, 2001; Pottenger *et al.*, 2000). However, the relationship between the structure and activity of BPA derivatives, including metabolites, remains to be fully understood.

In this report, endocrine-disrupting activity, i.e., estrogenic, anti-estrogenic, androgenic, anti-androgenic, thyroid hormonal, and anti-thyroid hormonal activities of BPA and related compounds were examined using hormone-responsive reporter assays: the human breast cancer cell-line MCF-7 for estrogenic activity, the mouse fibroblast cell line NIH3T3 for androgenic activity, and the pituitary cell line GH3 for thyroid hormonal activity. Twenty BPA derivatives were tested in this study (Fig. 1). We found that BPA and some of its derivatives exhibited estrogenic as well as anti-androgenic activity. TBBPA, TCBPA, TMBPA, and DMBPA showed significant thyroid hormonal activity. The structure-activity relationship of BPA derivatives is discussed.

MATERIALS AND METHODS

Chemicals. TBBPA, TCBPA, BPA, BPAD, BPB, BPF, BPAF, BPS, diphenylmethane (DPM), 4-hydroxydiphenylmethane (HDM), DMBPA, TMBPA, *p*-isopropylphenol (IPP), 1,1-bis-(4-hydroxyphenyl)cyclohexane (BPCH), α,α'-bis-(4-hydroxyphenyl)-1,4-diisopropylbenzene (BPDB), dihydrotestosterone (DHT), and flutamide were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), 2-(4-hydroxyphenyl)-2-phenylpropane (HPP) from Nacalai Tesque, Inc. (Kyoto, Japan), 2-(3,4-dihydroxyphenyl)-2-phenylpropane (BPA catechol) from Wako Pure Chemical Co. Ltd., (Osaka, Japan), 2,2-diphenylpropane (DPP) from Aldrich Chemical Co. (Milwaukee, WI), and L-3,5,3'-triiodothyronine (T_3) and 17-β-estradiol (E2) from Sigma Chemical Co. (St. Louis, MO). 2,2-bis-(4-Hydroxyphenyl)-1-propanol (BPA ol) and 2,2-bis-(4-hydroxyphenyl)-1-propionic acid (BPA carboxylic acid) were synthesized by the methods of Spivack *et al.* (1994).

Cell culture. Human breast cancer cell-line MCF-7 cells were maintained in DMEM (Sigma Chemical Co.) containing penicillin and streptomycin with 5% fetal bovine serum (FBS; Life Technologies, Rockville, MD). Rat pituitary cancer cell-line GH3 cells were maintained in DMEM/F12 mixed medium (Sigma Chemical Co.) containing penicillin and streptomycin with 8% horse serum (Life Technologies) and 2% FBS. Mouse fibroblast cell-line NIH3T3 cells were maintained in DMEM (Sigma Chemical Co.) containing penicillin and streptomycin with 5% calf serum (Life Technologies).

Assay of estrogenic activity of BPA and related compounds. ERE-luciferase reporter assay using MCF-7 cells was performed according to the previously reported method (Kitamura *et al.*, 2003a). Briefly, transient transfections in MCF-7 cells were performed using Transfast (Promega Co., Madison, WI), according to the manufacturer's protocol. Transfections were done in 48-well plates at 2×10^4 cells/well with 0.3 μg of p(ERE)$_3$-SV40-luc

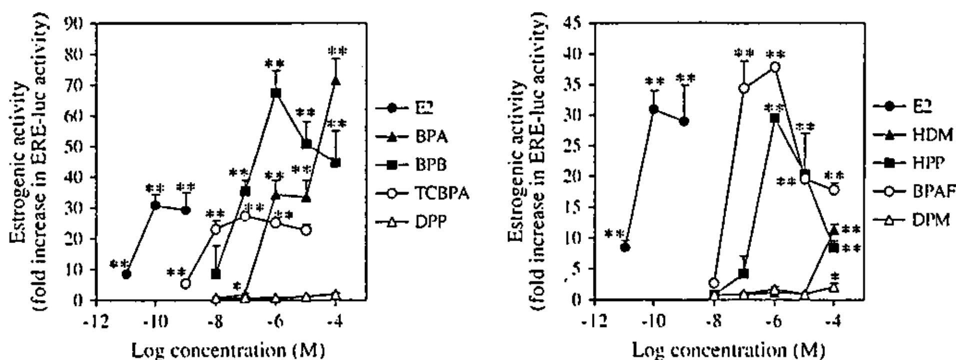

FIG. 2. Estrogenic activity of bisphenol A (BPA) and related compounds in ERE-luciferase reporter assay with MCF-7 cells. Each bar represents the mean ± SD of four experiments. Estrogenic activity was expressed as a relative activity with respect to the control using MCF-7 cells. BPB: bisphenol B, BPAF: bisphenol AF, TCBPA: tetrachlorobisphenol A, 4-HDM: 4-hydroxydiphenylmethane, HPP: 2-(4-hydroxyphenyl)-2-phenylpropane, DPM: diphenylmethane, DPP: 2,2-diphenylpropane, E2: 17-β-estradiol. $*p < 0.05$, $**p < 0.01$ compared with control.

and 2 ng of phRL-CMV (Promega Co.) as an internal standard (Sugihara *et al.*, 2000). Twenty-four hours after addition of the sample (final concentration, 10^{-4} – 10^{-9} M), the assay was performed with a Dual Luciferase assay kit (Promega Co.). Firefly luciferase reporter activity was normalized to renilla luciferase activity from phRL-CMV, to control for the cytotoxic effects of compounds, as well as differences in transfection efficiency between culture wells. For the assay of anti-estrogens, the inhibitory effect of BPA and related compounds on the estrogenic activity of E2 at the concentration of 1×10^{-10} M was examined.

Assay of androgenic activity of BPA and related compounds. Assay of androgenic activity was performed by means of ARE-luciferase reporter assay using NIH3T3 cells without expressing AR. Cells were maintained in phenol red-free DMEM (Sigma Chemical Co.) containing penicillin, streptomycin, and dextran-charcoal-treated calf serum for 2–3 days. Transient transfections in NIH3T3 cells were performed using Transfast according to the manufacturer's protocol. Transfections were done in 48-well plates at 2×10^4 cells/well with 0.3 μg of $p(ARE)_2$-luc, 0.05 μg of pSG5-hAR, and 2 ng of phRL-CMV as an internal standard (Kitamura *et al.*, 2003c). Twenty-four hours after addition of the sample (final concentration, 10^{-4} – 10^{-8} M) dissolved in 10 μl of ethanol, the assay was performed with a Dual Luciferase assay kit according to the manufacturer's protocol. Firefly luciferase reporter activity was normalized to renilla luciferase activity from phRL-CMV. For the assay of anti-androgenic activity, the inhibitory effect of BPA and related compounds on the androgenic activity of 1×10^{-10} or 1×10^{-11} M DHT was examined.

Assay of thyroid hormonal activity of BPA and related compounds. Assay of thyroid hormonal activity was performed by measuring the induction of growth hormone production in GH3 cells as previously reported (Kitamura *et al.*, 2002). Briefly, the cells were seeded in 24-well plates at 1×10^4 cells/well and chemicals were added the next day. Two days later, growth hormone in the culture medium was measured. For the assay of anti-thyroid hormonal activity, the inhibitory effect of BPA and related compounds on the activity of 1×10^{-7} or 1×10^{-8} M T3 was examined.

Assay of estrogenic activity in vivo *(uterotrophic assay).* B6C3F1 female mice obtained from Charles River Co. (Kanagawa, Japan) were used. They were surgically ovariectomized at four weeks old. At the age of eight weeks, they were divided into 14 groups each consisting of five animals. The mice were treated once a day for three days with ip doses of 0.2 ml of vehicle (Panacete 810, Nippon Oils and Fats Co., Ltd., Tokyo, Japan), E2 (50 μg/kg/day), TCBPA, TBBPA, or BPA (20, 100, 300, or 500 mg/kg/day). Animals were sacrificed under anesthesia and the uterus was dissected and weighed.

Data analysis. Multiple comparisons were made by ANOVA followed by Scheffe's test. EC50 values and IC50 values were calculated by fitting data to the logistic equation.

RESULTS

Estrogenic Activity of BPA and Related Compounds

Estrogenic activity of BPA and related compounds was examined using ERE-luciferase reporter assay in MCF-7 cells. BPA, BPB, BPF, BPA ol, BPA carboxylic acid, HPP, HDM, TMBPA, BPA catechol, DDM, TBBPA, DMBPA, and TCBPA all exhibited estrogenic activity in the estrogen screening assay, but the activities varied markedly from compound to compound. TCBPA, BPAF, BPB, and HPP showed significant estrogenic activity in the concentration range of 1×10^{-7} (1×10^{-8}

TABLE 1
EC50 Values of Bisphenol Derivatives in MCF-7 Estrogen Luciferase Reporter Assay

	EC50 (μM)
TCBPA	0.02
BPAF	0.05
BPB	0.07
HPP	0.15
BPCH	0.21
HDM	0.32
DMBPA	0.42
BPA	0.63
TMBPA	0.73
BPAD	0.91
BPF	1.0
BPS	1.1
BPA acid	1.1
BPA catecol	1.8
BPA ol	11
TBBPA	19
IPP	36
DPP	>1000
DPM	>1000
BPD	>1000
E_2	8.6×10^{-6}