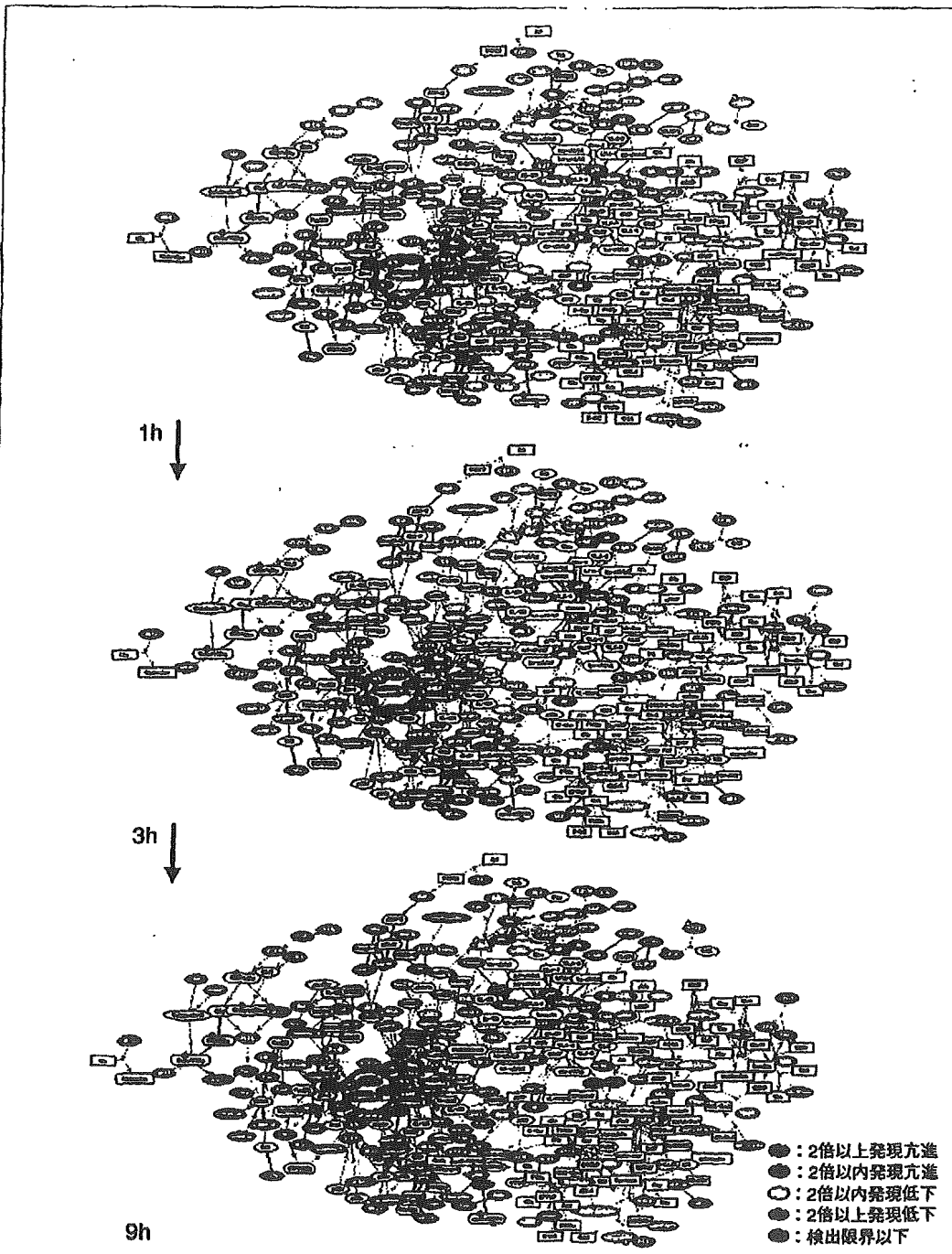
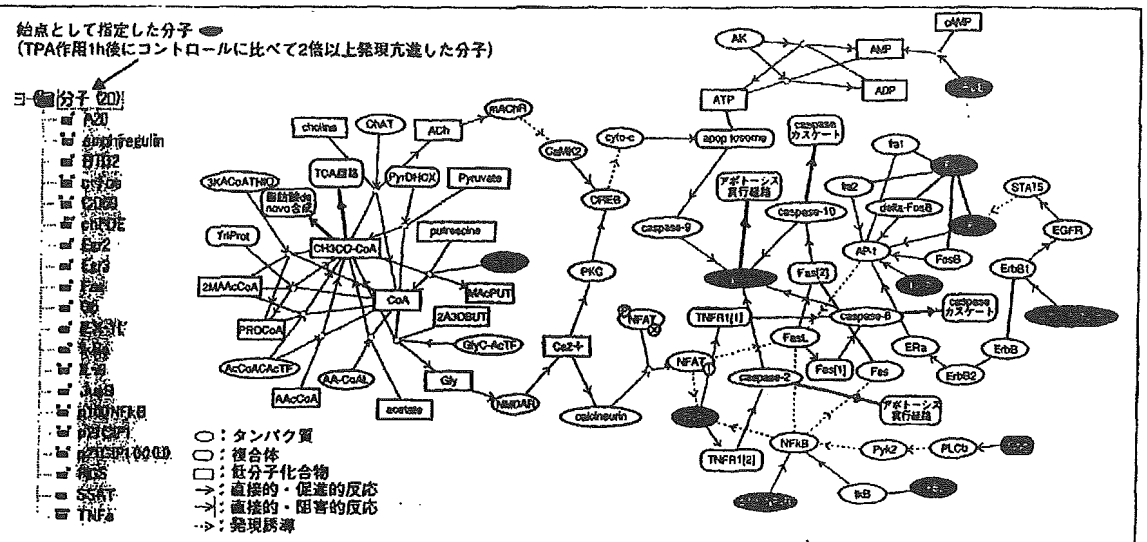
図❾　caspase-3を介したアポトーシス誘導のネットワーク（OR演算による）



HL-60においてTPA処理によりコントロールに比べて2倍以上発現亢進した分子群（DNAマイクロアレイデータ：AffymetrixR U95A）を始点に，アポトーシス関与分子caspase-3（GO:0006915）を終点に指定して描かせた3つのタイムコース（1，3，9時間）によるネットワークの全部分をOR演算によりマージした。各時間における数値データ（コントロールと比べた遺伝子の発現量比）をカラーリングすると，時間による発現量の推移が可視化できる。黒丸で囲んだ分子がcaspase-3。（グラビア頁参照）

（データおよびデータ処理法（FUMI理論）提供：国立医薬品食品衛生研究所）

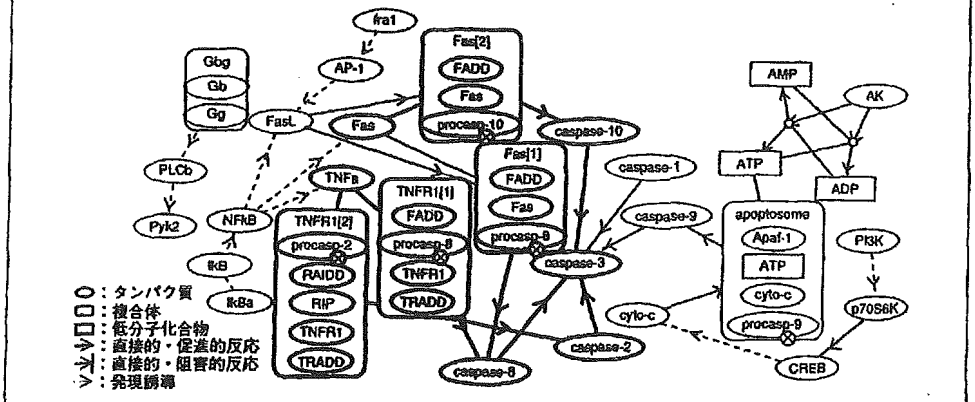
図❻ TPA処理1時間後に発現亢進した分子群よりcaspase-3に至るネットワーク



HL-60においてTPA処理1時間後にコントロールに比べて2倍以上発現亢進した分子群を抽出し（図左側にリスト表示），これらの分子を始点として，caspase-3に至るネットワークを描かせた（始点分子：赤，終点分子：青）。

（グラビア頁参照）

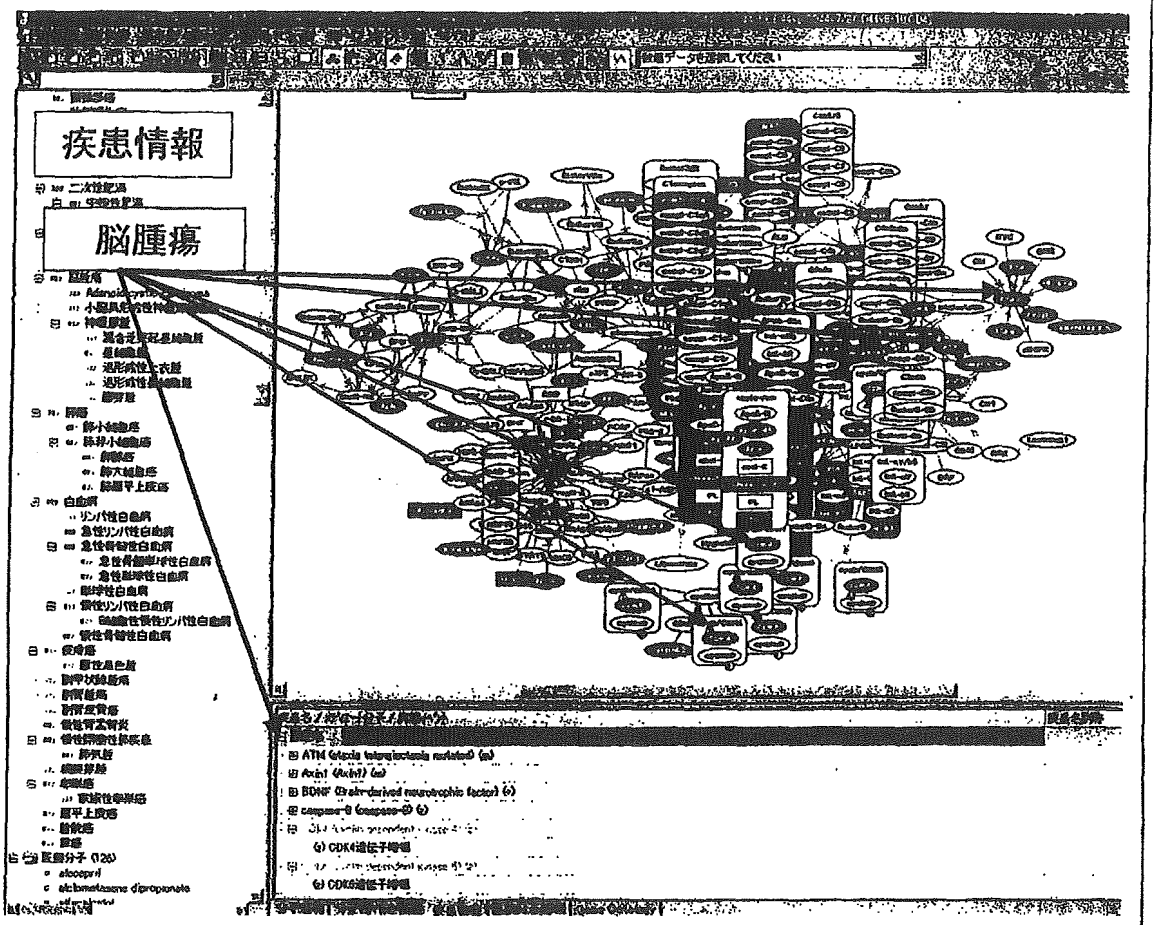
図❺ caspase-3を介したアポトーシス誘導のネットワーク（AND演算による）



HL-60においてTPA処理によりコントロールに比べて2倍以上発現亢進した分子群を始点に，caspase-3を終点に指定して描かせた3つのタイムコース（1，3，9時間）による3つのネットワークの共通部分をAND演算により抽出した。

をAND演算を用いて抽出するとよい（図❺）。この解析により，TPA作用下HL-60では，TNF receptor, TNFαまたはFasを介し，caspase-3に至り，アポトーシスが誘導されるメカニズムが示唆された。TPAによりTNFα，Fasを介してU937, HL-60にアポトーシスが誘導されるという報告があるため[97]，この系においても同様な経路によってアポトーシスが誘導される可能性はあり，さらに解析を続けることにより未知の経路も見出されるかもしれない。しかし，このパスウェイが実際に関与しているか否かは，タンパクレベルの実験を行わなければ確認できない。

図⑥　ヒト脳腫瘍で発現亢進したタンパク間のネットワークと脳腫瘍関連分子



プロテオミクス解析により得られたヒト脳腫瘍で発現亢進した分子間でネットワークを描かせた（図はその一部，プロテオミクスデータ：熊本大学医学部荒木令江先生）。色づけされた分子は疾患関連分子（KeyMolnetでは疾患メディエート分子と呼ぶ）であり，実際のKeyMolnetでは，さらにこの上に脳腫瘍関連因子がハイライトされる。下段の情報ビューではこれらの分子とリンクした疾患や医薬品などの詳細情報が得られ，疾患メディエート分子，医薬品のターゲット分子からも分子ネットワークにジャンプでき，ネットワークを生成できる。このようにKeyMolnetでは分子ネットワーク-疾患-医薬品の間を縦横無尽に行き来できる。

Ⅲ．プロテオームデータ解析への応用

mRNAとタンパクの発現は，同一条件の同じサンプルで解析しても決して一致などしない。KeyMolnetでは前述したDNAマイクロアレイデータと同様にプロテオミクスデータも解析できる（図⑥）。ここでは，ヒト脳腫瘍において発現亢進したタンパク分子間で描かせたネットワークに，脳腫瘍に関与することが知られている分子（KeyMolnetでは疾患メディエート分子と呼ぶ）を表示させた。このように既存の疾患のデータと組み合わせたり，あるいはゲノムデータと比較しながらネットワーク解析を行うことができる。

転写因子を介した複雑なネットワークにおける生体分子の機能解析にはトランスクリプトームは重要であるが，それのみでは，特にシグナル伝達経路は解析できない。生体を総合的に捉え，生命現象を理解するには，あらゆる情報の統合と，そ

こから真実を取り出すことのできるデータマイニングツールが必要であり，ゲノム情報，プロテオーム情報，さらにはタンパクの修飾情報までをも同一の土俵で扱えるプラットフォームが必須となってくる。シグナル伝達の解析ではタンパク量の増減だけでなく，リン酸化などのタンパクの修飾状態を調べることも重要である。医薬分子設計研究所ではタンパク質の変動や修飾状態に注目した，分子の機能予測，疾患関連物質の特定，drug targetの選定およびバリデーションを行うためのKeyTarget法[用語3]を展開中であり，KeyMolnetとリンクさせることにより独自の解析を試みている。

おわりに

今回紹介したゲノミクス，プロテオミクスデータは同一条件，同一サンプルによるものではないが，前述したように，生体を総体として理解するためには，そしてそれらを社会に役立てるための疾患メカニズム解析，医薬品の開発へと結びつけるためには，さらなる情報の統合が必要であり，KeyMolentではそれが可能であると考える。実際，現在これらの情報を組み合わせた総合的な解析が進行中である。あらゆる情報を統合できたうえで初めて網羅的解析の真価が発揮され，論理的・効率的な疾患メカニズム研究およびその標的薬の開発への貢献が期待できるであろう。

※KeyMolnetはバージョンアップを重ねているため，コンテンツ，機能とも順次更新されている。

用語解説

1．**データマイニング**：マイニング（mining）は，採鉱，採掘という意味であり，データマイニングとは鉱山であたかも金脈を掘り当てるがごとく，膨大なデータの山から意味のある，あるいは価値のある情報，相関関係などを抽出すること。
2．**テキストマイニング**：テキスト（自然文,ライフサイエンスの分野では多くの場合，原著論文のアブストラクトがこの対象となる）を語句に分解し，統計処理を行うデータマイニング手法を応用したもの。
3．**KeyTarget法**：薬物が生体（細胞，組織，個体）に与える影響を，生体分子ネットワークから適宜選んだ既知タンパク分子群の発現量・修飾量の変化として検出し，KeyMolnetで分析するIMMD独自の方法。

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ACUTE, SUBCHRONIC AND CHRONIC TOXICITY STUDIES OF A SYNTHETIC ANTIOXIDANT, 2,2'-ISOBUTYLIDENEBIS(4,6-DIMETHYLPHENOL) IN RATS

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ABSTRACT — General toxicity studies on 2,2'-isobutylidenebis(4,6-dimethylphenol)(IBBMP) were conducted using male and female Wistar rats.

In the acute test, the oral LD50 values were 119 mg/kg BW in males and 103 mg/kg BW in females. Hypersensitivity, loss of righting reflex and abdominal position were observed.

In the subchronic test, rats were fed a diet containing IBBMP at levels of 0, 20, 100 or 500 ppm for 13 weeks with interim sacrifice at 4 weeks (equal to 0, 1.1, 5.5 or 27.9 mg/kg BW/day in males and 0, 1.1, 5.9 or 29.6 mg/kg BW/day in females). In both sexes, there were no changes in general condition, body weight gains and food intakes in all groups. No deaths were observed in all groups. Significant increase in AST was observed in 500 ppm males at Week 4. However, the change was not observed at Week 13. Slight but significant decreases in creatinine were also observed in 100 ppm females at Week 13 and 500 ppm males and females at Weeks 4 and 13. Total cholesterol (T-CHO) was significantly elevated in females of the 500 ppm group at Weeks 4 and 13. Absolute and relative liver weights were increased in 500 ppm of both sexes at Week 4. In females, the increases were also observed at Week 13. However, no remarkable histopathological findings were observed in all treated groups.

In the chronic test, rats were fed a diet containing IBBMP at levels of 0, 100, 500 and 1500 ppm for 18 months with interim sacrifices at 6 and 12 months (equal to 0, 3.8, 19.4 or 59.4 mg/kg BW/day in males and 0, 4.3, 20.9 or 67.5 mg/kg BW/day in females). No remarkable changes in general appearance were observed in any rats. Body weight gains, food intakes and survival rates in all treated animals were comparable to those of the control. No remarkable changes in the hematological parameters were observed. T-CHO was significantly elevated in females of the 1500 ppm groups throughout the experiment. Significant increases or tendencies for increase in relative liver weights were observed in the 500 and 1500 ppm animals of both sexes. Increased incidences of swelling in liver cells were observed in 1500 ppm males at 6 months and 1500 ppm females at 12 and 18 months. At 18 months, dose-dependent increases in thickness of basement membrane of renal tubules and Bowman's capsule and cell infiltration to the interstitium of the kidney were observed in males. Significant increases of hyaline cast and basophilic change were also observed in 1500 ppm males. In females, increased incidences of hyaline cast were observed at 500 ppm and higher at 18 months. No other toxicity was apparent. No neoplastic lesions that could be attributed to IBBMP were observed in any organs of either sex. From the result of the chronic toxicity test, the no-observed-adverse-effect level (NOAEL) for IBBMP was concluded to be 100 ppm in the diet (4.26 mg/kg BW/day) in female rats on the basis of induction of hyaline cast in renal tubules at 500 ppm, whereas, in males, only a lowest-observed-adverse-effect level (LOAEL) was given as 100 ppm (3.84 mg/kg BW/day) on the basis of induction of thickening of basement membrane in renal tubules at 100 ppm.

KEY WORDS: 2,2'-isobutylidenebis(4,6-dimethylphenol), Acute toxicity test, Subchronic toxicity test, Chronic toxicity test

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INTRODUCTION

2,2'-Isobutylidenebis(4,6-dimethylphenol) (IBBMP) is one of the phenolic antioxidants that has been used in the rubber and plastics industries for protection against oxidative damage. IBBMP was not teratogenic in Wistar rats when tested at doses up to 45 mg/kg/day. The developmental toxicity of NOAEL was 45 mg/kg/day, the highest dose tested, and the NOAEL for maternal toxicity for 5 mg/kg/day, on the basis of toxic signs (tremor, startle reflex, salivation, involuntary urination, wheezing and nostril discharge), at 15 mg/kg/day (Ishii *et al.*, 1991). We previously reported that 2,2'-methylenebis-(4-methyl-6-*tert*-butylphenol) (MBMBP) (CAS REGISTRY No.:119-47-1), which has a structural resemblance to IBBMP (Fig. 1), induces testicular atrophy in rats when given for 18 months at 0.1% in the diet (42.3 mg/kg BW/day) (Takagi *et al.*, 1994). Meanwhile, complete toxicological studies (short-term and long-term test) of IBBMP have not been reported as yet. The present acute, subchronic and chronic toxicity studies were therefore conducted to elucidate the hazard potential of this environmentally important agent.

MATERIALS AND METHODS

Chemicals

IBBMP (CAS REGISTRY No.; 33145-10-7) (Technical grade) was obtained from Bayer Japan Ltd. All reagents used for hematological and biochemical analyses were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan).

Experimental animals and diets

Specific pathogen-free Wistar male and female

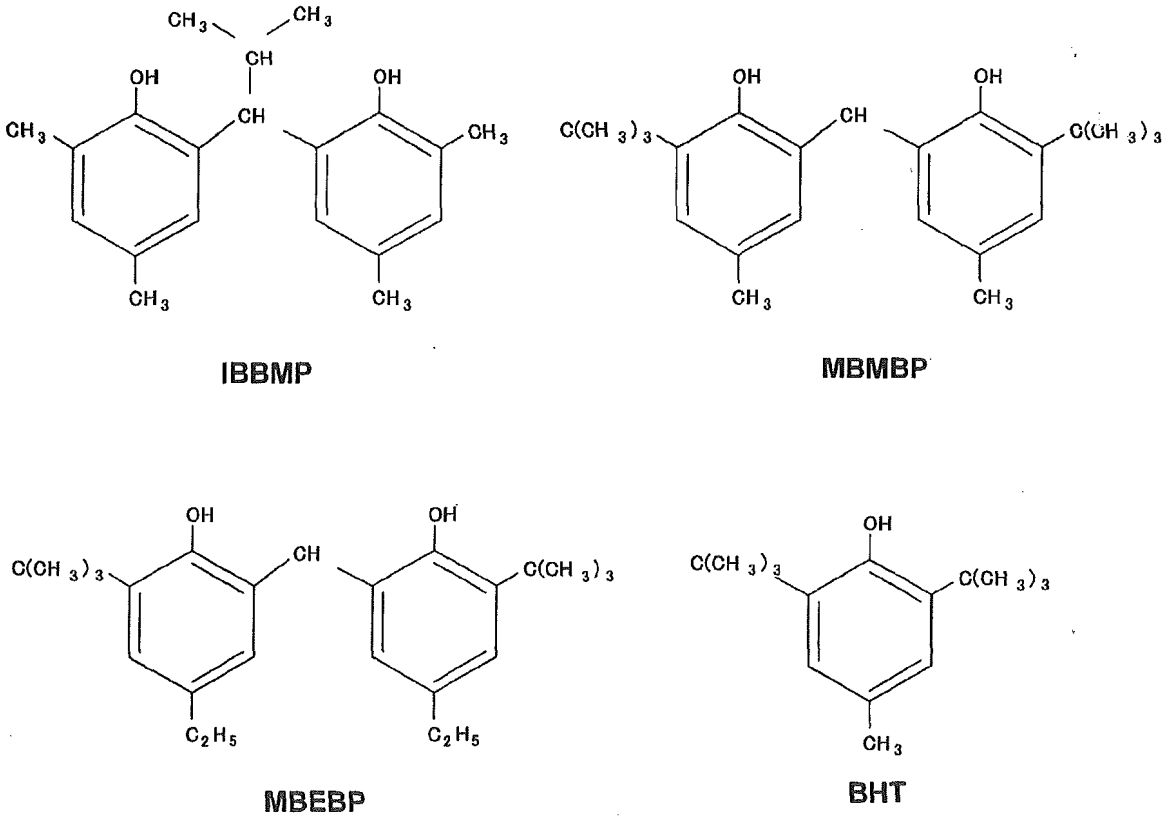

Fig. 1. Comparison of the chemical structures of four phenolic antioxidants.

rats (4 weeks old) were purchased from SLC Co. (Hamamatsu, Japan) and acclimated for 1 week prior to initiation of the tests. The basal diet powder was purchased from Funabashi Farm (F-2, Funabashi, Japan) and IBBMP was mixed into the diet (subchronic and chronic tests) prior to pelleting at the desired concentrations and stored at 4°C until use.

Housing conditions

The animal room was maintained at a temperature of 24 ± 1°C and 55 ± 5% humidity with a 12 hr light/dark cycle. Rats were housed in plastic cages (4-5 rats/cage) using chip bedding in the acute, subchronic and chronic studies.

Experimental design and performance

1. Acute toxicity test

Five rats (body weight: males, 96-122 g; females, 83-105 g) per group of each sex were fasted for 16hr before and 6hr after administration. IBBMP suspended in olive oil (2% w/v) was orally administered at the dose of 75-223.9 mg/kg BW in males and 75-155.5 mg/kg BW in females. Toxic signs and mortality were monitored up to Day 14 when the test was terminated. LD50 value was calculated by the Litchfield-Wilcoxon method.

2. Subchronic feeding study

Groups of 10 rats of each sex were given pellets containing 0 (control), 0, 20, 100 or 500 ppm IBBMP. The body weights and food consumption values were recorded weekly. During the entire course of the study, the rats were observed daily. Five randomly chosen rats from each group were examined at Week 4 as an intermittent study and the experiment was terminated at Week 13. Hematological and serum biochemical examinations were conducted after 16 hrs of starvation. All animals were studied for histological changes.

3. Chronic feeding study

Groups of 30 rats of each sex were given pellets containing 0 (control), 100, 500 or 1500 ppm IBBMP. General condition was monitored daily and body weights and food consumption values were recorded monthly throughout the experiment. Groups of 5 animals at months 6, 12 and 18 underwent hematological and serum biochemical examinations after 16 hrs of starvation. All animals (5 animals/group at Months 6 and 12, and all surviving animals at Month 18 as well as those dying during the experiment) were studied for histological changes.

Survival rates in the subchronic and chronic toxicity tests were assessed using the life table technique (Sachs, 1959).

Clinical examination

In the subchronic and chronic toxicity tests, the following examinations were conducted. Blood was collected from the orbital venous plexus under ether anesthesia and the hematological parameters, such as red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean hemoglobin concentration (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelets (PLT) and white blood cells (WBC) were assessed with a Sysmex M-2000 (SYSMEX Co. Japan). Differential white blood cell counts were made with a MICROX CELL ANAZYZER/HEG-120A (OMRON Co., Japan).

Serum biochemical assessments were conducted for 24 items; total protein (T-PRO), albumin (ALB), albumin-globulin ratio (A/G), blood urea nitrogen (BUN), creatinine (CRN), glucose (GLU), non-esterified fatty acids (NEFA), phospholipids (PL), triglycerides (T-GLY), total cholesterol (T-CHO), free cholesterol (F-CHO), alkaline phosphatase (ALP), amylase (AMY) (subchronic test only), cholinesterase (CHE)(subchronic test only), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), leucine aminopeptidase (LAP) (subchronic test only), calcium (Ca), inorganic phosphorus (P), sodium (Na), potassium (K), chloride (Cl) and magnesium (Mg) using a Automatic Analyzer 7150 type (Hitachi Ltd., Japan).

At autopsy, the weights of the brain, heart, lungs, liver, kidneys, spleen, adrenals, testes, ovaries, pituitary and thyroid glands were measured. The above-mentioned organs and the salivary glands, esophagus, stomach, small and large intestine, pancreas, urinary bladder, seminal vesicles, epididymis, sciatic nerve, uterus, prostate, mesenteric lymph nodes, thymus, spinal cord, skeletal muscle and bone marrow (femur and sternum) were fixed in 10% buffered formalin solution for routine histological processing. Paraffin sections were stained with hematoxylin and eosin for histopathological examination.

Statistical analysis

All quantitative data except for the histopathological findings were statistically analyzed by one-way analysis of variance (ANOVA) techniques with Dunnett's (Dunnett, 1964) or Scheffe's (Scheffe, 1953)

multiple comparison procedures. Histopathological data were statistically analyzed with the Fisher's exact probability test (Fisher, 1973) or Mann-Whitney U-test (Mann and Whitney, 1947).

RESULTS

Acute toxicity

IBBMP was moderately toxic. The oral LD50 values for rats were 119 mg/kg BW in males and 103 mg/kg BW in females. Hypersensitivity, loss of righting reflex and abdominal position were observed. All deaths occurred in rats of either sex during 6 to 24 hrs after administration.

Subchronic feeding study

1. General symptoms

In both sexes, there were no changes in general condition, body weight gains and food intakes in all groups (data not shown). No deaths were observed in all groups. The calculated mean IBBMP intakes in rats fed containing 0, 20, 100 or 500 ppm IBBMP for 13 weeks are 0, 1.1, 5.5 or 27.9 mg/kg BW/day in males and 0, 1.1, 5.9 or 29.6 mg/kg BW/day in females.

2. Hematological and serum biochemical examinations

For the hematological and serum biochemical analyses, only parameters demonstrating significant changes in either sex are listed in Table 1. Significant

Table 1. Hematological and serum biochemical findings for rats fed a diet containing IBBMP for 13 weeks.

Weeks	Group (ppm)	Hb (g/dl)	MCH (pg)	MCHC (g/dl)	CRN (mg/dl)	T-CHO (mg/dl)	F-CHO (mg/dl)	AST (mU/l)	Na (mEq/l)
Males	0	16.4	17.6	32.5	0.45	64	10.3	80	148
		± 0.5	± 0.2	± 0.2	± 0.01	± 5	± 1.1	± 7	± 0
	20	16.3	17.6	32.7	0.44	65	10.2	86	146
		± 0.6	± 0.2	± 0.5	± 0.02	± 6	± 1.6	± 13	± 2
4	100	16.3	17.6	32.6	0.45	64	8.9	79	145*
		± 0.2	± 0.2	± 0.3	± 0.02	± 5	± 1.9	± 10	± 1
	500	16.3	17.6	32.5	0.41*	65	10.5	98*	145**
		± 0.5	± 0.3	± 0.3	± 0.03	± 4	± 1.8	± 7	± 1
	0	15.8	16.8	32.3	0.45	93	23.8	83	135
		± 0.7	± 0.3	± 0.4	± 0.03	± 12	± 3.5	± 15	± 0
	20	15.9	16.5	31.5	0.47	90	26.8	95	136
		± 0.4	± 0.3	± 0.6	± 0.03	± 8	± 3.9	± 28	± 1
13	100	15.9	17.2	32.3	0.59	92	21.4	90	136
		± 0.4	± 0.2	± 0.6	± 0.35	± 10	± 1.2	± 19	± 0
	500	16.4	16.9	31.4	0.38*	90	18.5*	79	137**
		± 0.8	± 0.5	± 0.8	± 0.03	± 7	± 2.0	± 16	± 1
Females	0	16.8	18.2	33.4	0.52	85	14.3	92	147
		± 0.2	± 0.3	± 0.5	± 0.04	± 10	± 2.7	± 6	± 1
	20	16.8	18.3	33.3	0.45*	96	16	90	147
		± 0.6	± 0.2	± 0.5	± 0.07	± 13	± 2.5	± 6	± 1
4	100	16.8	18.3	33.6	0.42**	85	13.9	99	146
		± 0.5	± 0.7	± 1.0	± 0.02	± 5	± 1.7	± 10	± 2
	500	16	18.5	33.6	0.43*	101*	16.7	91	147
		± 0.6	± 0.4	± 0.6	± 0.03	± 7	± 1.6	± 9	± 2
	0	17.6	19	32.9	0.51	122	28.6	69	138
		± 0.8	± 0.9	± 1.1	± 0.05	± 11	± 3.6	± 7	± 1
	20	18.7	21.4	36.4*	0.49	117	30.5	79	138
		± 0.9	± 0.6	± 1.2	± 0.04	± 15	± 7.7	± 9	± 1
13	100	20.3**	22*	37.6**	0.44*	131	32	70	138
		± 0.4	± 0.6	± 1.1	± 0.02	± 5	± 2.6	± 4	± 1
	500	20.4**	22.7**	38.9**	0.47	143*	32.5	66	136
		± 1.8	± 2.1	± 3.0	± 0.03	± 9	± 2.6	± 7	± 2

Values are the means ± S.D. for data from 5 rats.
Significantly different from control; *p<0.05, **p<0.01.

increases of Hb, MCH and MCHC were observed in 100 and 500 ppm females at Week 13. Significant increase of AST was observed in 500 ppm males at Week 4. However, the change was not observed at Week 13. Slight but significant decreases of CRN were also observed in 100 ppm females at Week 13 and 500 ppm males and females at Weeks 4 and 13. T-CHO was significantly elevated in females of the 500 ppm group at Week 4 and thereafter. Na level was slightly decreased in the 100 and 500 ppm males at Week 4 and increased in the 500 ppm males at Week 13.

3. Organ weights

Only organs that showed significant changes throughout the study are listed in Table 2. Absolute and relative liver weights were increased in 500 ppm of both sexes at Week 4. In females, increases of absolute and relative liver weights were also observed at Week 13.

4. Histopathological examination

In all treated groups, no remarkable histopathological changes were observed in any organs including liver (data not shown).

Chronic feeding study

Because of the no remarkable histopathological changes of the highest-dose group (500 ppm in diet) in the 13-week study, doses selected for the 18-month study were 100, 500 and 1500 ppm in the diet.

1. General appearance, mortality, body weight gain

No remarkable changes in general appearance were observed in any rats. Body weight gains, food intakes and survival rates in all treated animals were comparable to those of control (data not shown). The calculated mean IBBMP intakes in rats fed containing 0, 100, 500 or 1500 ppm IBBMP for 18 months are 0, 3.8, 19.4 or 59.4 mg/kg BW/day in males and 0, 4.3, 20.9 or 67.5 mg/kg BW/day in females.

2. Hematological and serum biochemical findings

In the hematological and serum biochemical analyses, T-CHO was significantly elevated in females of the 1500 ppm groups throughout the experimental period (Table 3). Several other parameters demonstrated significant alteration. However, none appeared to be of toxicological significance, since they did not show the same tendency throughout the experimental period and/or the degrees of change were very small.

3. Organ weights

Significant increases or tendencies for increase in relative liver weights were observed in the 500 and 1500 ppm animals of both sexes (Table 4). Significant

Table 2. Liver weights for rats fed a diet containing IBBMP for 13 weeks.

Weeks		Group (ppm)	Absolute liver weight (g)	Relative liver weights (g%)
Males	4	0	7.28 ± 0.36	3.01 ± 0.12
		20	7.74 ± 0.35	3.20 ± 0.16
		100	7.67 ± 0.34	3.06 ± 0.04
		500	8.17 ± 0.47**	3.27 ± 0.15*
	13	0	9.85 ± 0.99	2.71 ± 0.13
		20	9.51 ± 0.77	2.62 ± 0.10
		100	9.64 ± 0.45	2.66 ± 0.08
		500	10.54 ± 0.57	2.79 ± 0.10
Females	4	0	4.12 ± 0.20	2.72 ± 0.06
		20	4.27 ± 0.40	2.76 ± 0.12
		100	4.46 ± 0.18	2.80 ± 0.05
		500	5.17 ± 0.37**	3.09 ± 0.28**
	13	0	4.36 ± 0.26	2.14 ± 0.08
		20	4.55 ± 0.23	2.31 ± 0.14
		100	4.84 ± 0.39*	2.31 ± 0.10
		500	4.92 ± 0.35*	2.49 ± 0.11**

Values are the means ± S.D. for data from 5 rats.
Significantly different from control; *p<0.05, **p<0.01.

increases of absolute and relative kidney weights were observed in the 1500 ppm males at 12 months. No remarkable changes in other organs were observed in any treated groups for either sex.

4. Histopathological examination

Histopathological lesions were only observed in the liver and kidney of both sexes. Significant increased incidences of swelling in liver cells and basophilic change, thickening of basement membrane in renal tubules were observed in 1500 ppm males at 6 months. Increased tendency of eosinophilic body renal tubules in the 1500 ppm males was also noted (Table 5, 8). At 12 months, increased incidence of swelling in liver cells was observed only in 1500 ppm females. (Table 6, 8). At 18 months, dose-dependent increase in thickness of basement membrane of renal tubules and Bowman's capsule and cell infiltration to Interstitium

Table 3. Changes of T-CHO levels (mg/dl) in serum of female rats fed a diet containing IBBMP for 18 months.

	Group				
Months	0	100	500	1500	ppm
6	139 ± 17	131 ± 10	158 ± 10	168 ± 17*	
12	142 ± 15	157 ± 15	154 ± 16	204 ± 31**	
18	150 ± 20	145 ± 20	171 ± 16	229 ± 85*	

Values are the means ± S.D. for data from 5 rats.
Significantly different from control; *p<0.05, **p<0.01.

Table 4. Body and organ weights for rats fed a diet containing IBBMP for 18 months.

Months	Group (ppm)	Body weight (g)	Absolute liver wt (g)	Relative liver wt (g%)	Absolute kidney wt (g)	Relative kidney wt (g%)
Males	0	414 ± 31	9.27 ± 0.81	2.24 ± 0.04	2.01 ± 0.25	0.48 ± 0.04
6	100	415 ± 19	9.87 ± 0.55	2.38 ± 0.07	2.07 ± 0.13	0.50 ± 0.02
	500	419 ± 20	10.06 ± 0.53	2.40 ± 0.13*	2.11 ± 0.15	0.50 ± 0.03
	1500	420 ± 9	12.12 ± 0.55**	2.89 ± 0.12**	2.22 ± 0.18	0.53 ± 0.04
12	0	477 ± 11	10.17 ± 0.28	2.13 ± 0.10	2.20 ± 0.15	0.46 ± 0.03
	100	475 ± 31	10.58 ± 0.55	2.23 ± 0.07	2.20 ± 0.13	0.46 ± 0.02
	500	484 ± 24	11.00 ± 0.63	2.28 ± 0.09	2.32 ± 0.16	0.48 ± 0.04
	1500	473 ± 24	12.69 ± 1.02**	2.68 ± 0.09**	2.60 ± 0.21**	0.55 ± 0.07*
18	0	508 ± 28	13.23 ± 2.20	2.61 ± 0.45	2.63 ± 0.33	0.52 ± 0.07
	100	499 ± 18	11.00 ± 1.06	2.20 ± 0.19	2.36 ± 0.11	0.47 ± 0.02
	500	503 ± 30	12.26 ± 1.71	2.44 ± 0.27	2.61 ± 0.32	0.52 ± 0.06
	1500	491 ± 23	14.16 ± 1.14	2.89 ± 0.33	2.67 ± 0.26	0.55 ± 0.07
Females	0	219 ± 16	5.04 ± 0.69	2.30 ± 0.19	1.16 ± 0.07	0.53 ± 0.04
6	100	219 ± 11	4.66 ± 0.21	2.14 ± 0.14	1.20 ± 0.10	0.55 ± 0.03
	500	222 ± 11	6.22 ± 0.86*	2.79 ± 0.26*	1.27 ± 0.11	0.57 ± 0.02
	1500	221 ± 13	6.61 ± 0.51**	3.00 ± 0.35**	1.24 ± 0.04	0.56 ± 0.04
12	0	270 ± 26	5.91 ± 0.51	2.19 ± 0.09	1.40 ± 0.11	0.52 ± 0.04
	100	284 ± 43	6.09 ± 0.55	2.17 ± 0.22	1.35 ± 0.07	0.48 ± 0.06
	500	273 ± 23	6.71 ± 0.63	2.47 ± 0.38	1.41 ± 0.13	0.52 ± 0.06
	1500	273 ± 30	7.94 ± 1.31**	2.91 ± 0.36**	1.52 ± 0.17	0.56 ± 0.06
18	0	328 ± 26	6.75 ± 0.61	2.06 ± 0.06	1.55 ± 0.19	0.47 ± 0.04
	100	331 ± 20	6.52 ± 0.76	1.96 ± 0.14	1.52 ± 0.16	0.46 ± 0.03
	500	330 ± 24	7.00 ± 0.41	2.12 ± 0.10	1.53 ± 0.15	0.47 ± 0.07
	1500	298 ± 24	9.07 ± 2.18*	3.11 ± 1.04*	1.94 ± 0.74	0.67 ± 0.33

Values are the means ± S.D. for data from 5 rats.
Significantly different from control; *p<0.05, **p<0.01.

of kidney were observed in males. Significant increase of hyaline cast and basophilic change were observed in 1500 ppm males. In females, increased incidence of hyaline cast was observed at 500 ppm and higher at 18 months. Increased incidence of swelling in liver cells in 1500 ppm females was also observed at 18 months (Table 7, 8). No neoplastic lesions that could be attributed to IBBMP were observed in any organs of either sex.

DISCUSSION

In the present study, IBBMP showed moderate acute toxicity when administered orally to male and female Wistar rats. Hypersensitivity and loss of righting reflex induced by IBBMP suggest that the compound may be neurotoxic. However, no neurotoxic effects were observed in subchronic and chronic toxicity tests, indicating that neurological effects are limited to the high dose levels. A comparison of the toxicity of four phenolic antioxidants, based on our studies and those in the literature, are summarized in Table 9. BHT, which has a close structural resemblance to MBMBP (Fig. 1), and MBMBP have been reported to induce hemorrhagic death in rats (Takahashi and Hiraga, 1978; Takagi *et al.*, 1994). On the other hand, MBEBP (Fig.1) (5.0% in the diet) did not show any hemorrhagic effects on Wistar rats (Takagi *et al.*, 1992), or any decrease in prothrombin and Kaolin-PTT indices (Takahashi and Hiraga, 1981). Therefore, we speculated that a methyl moiety in the C4 position of MBMBP molecule might play an important role in the hemorrhagic action. However, in this experiment, neither death nor hemorrhage was observed in rats treated with IBBMP that has a methyl moiety in the C4 position. The results suggest that not only methyl moiety in

Table 5. Histopathological findings for rats fed a diet containing IBBMP for 6 months.

Organs	Sexes	Males				Females			
Findings	Dose (ppm)	0	100	500	1500	0	100	500	1500
No. of animals examined		5	5	5	5	5	5	5	5
Liver									
(liver cell)									
swelling	±	0	0	0	5	0	0	0	2
vacuolization	±	1	1	1	1	0	0	0	0
focal necrosis	±	0	1	0	0	0	1	0	0
microgranuloma	±	1	0	0	0	0	0	0	0
(Glisson's sheath)									
bile duct proliferation	±	0	1	0	0	0	0	0	0
cell infiltration	±	4	3	3	1	1	0	3	2
(sinusoid)	±								
congestion	+	0	0	0	0	0	0	0	1
		0	0	0	0	1	0	0	0
Kidney									
(tubules)									
eosinophilic body	±	3	5	2	1	0	0	0	0
	+	1	0	3	4	0	0	0	0
basophilic change	±	3	3	4	1	0	0	0	0
	+	0	0	0	4	0	0	0	0
hyaline cast	±	1	0	0	3	0	0	0	0
thickening of basement membrane	±	0	1	2	4	0	0	0	0
brown pigment deposition	±	3	2	3	3	5	4	5	4
cyst	+	0	0	1	0	0	0	0	0
congestion	+	0	0	0	0	1	0	0	0
(interstitium)									
cell infiltration	±	1	0	2	2	1	0	0	0

±: slight, +: mild, ++: marked.

the C4 position but also another chemical structure such as *tert*-butyl moiety would be necessary. The other remarkable effect of MBMBP was atrophy of the testicular tubules and decrease of spermatogenesis, accompanied by decrease in testis weight (Takagi *et al.*, 1996). We have previously reported that MBEBP (1% in the diet) treatment for 4 weeks caused atrophy of testicular tubules and decrease of spermatogenesis in the rats (Takagi *et al.*, 1992). The similarities in histological findings indicate that the testicular toxicity induced by both chemicals might be mediated by the same mechanisms. However, no testicular toxicity was observed in subchronic and chronic toxicity tests. Therefore, common molecule structures of MBMBP and MBEBP would be responsible for testicular toxicity. We previously showed that MBEBP exerts an uncoupling action on oxidative phosphorylation in isolated liver mitochondria and suggested that this might play a role in testicular toxicity (Takagi *et al.*, 1993). We have confirmed that MBMBP also demonstrates the same action to an even greater degree than MBEBP (Takagi, unpublished data). Interestingly, gossypol, which is a natural polyphenolic compound causing decrease of spermatogenesis and degeneration of tubu-

Table 6. Histopathological findings for rats fed a diet containing IBBMP for 12 months.

Organs	Sexes	Males				Females			
Findings	Dose (ppm)	0	100	500	1500	0	100	500	1500
	No. of animals examined	5	5	5	5	5	5	5	5
Liver									
(liver cell)									
clear cell foci	+	1	0	0	1	0	0	1	0
vacuolated foci	±	0	0	0	0	0	1	0	0
swelling	±	0	0	0	0	0	0	0	4
vacuolization	±	2	1	2	3	0	1	0	1
focal necrosis	±	3	0	1	0	0	0	0	0
	+	1	0	1	0	0	0	0	0
microgranuloma	±	0	1	0	0	0	0	0	1
brown pigment deposition	±	2	0	0	0	0	0	0	0
cystic degeneration	±	1	0	1	0	0	0	0	0
(Glisson's sheath)									
bile duct proliferation	±	3	3	4	5	2	1	1	2
cell infiltration	±	1	2	0	1	2	2	2	0
(sinusoid)	±								
congestion	+	0	0	0	0	0	0	0	1
extramedullary hematopoiesis	±	0	0	1	0	0	0	0	0
Kidney									
(tubules)									
eosinophilic body	±	3	1	1	4	0	0	0	0
	+	2	4	4	1	0	0	0	0
basophilic change	±	3	2	2	3	1	0	2	1
	+	2	3	3	2	0	0	0	0
hyaline cast	±	3	1	4	1	3	2	4	4
	+	1	0	0	4	0	0	0	1
thickening of basement membrane	±	3	4	1	3	0	0	0	1
brown pigment deposition	±	5	5	5	5	5	5	5	5
calcification	±	0	0	1	0	2	0	1	1
(interstitium)									
cell infiltration	±	2	3	3	3	0	0	1	1
fibrosis	±	0	0	1	0	0	0	0	0

±: slight, +: mild, ++: marked.

Table 7. Histopathological findings for rats fed a diet containing IBBMP for 18 months.

Organs / Findings		Sexes	Males				Females			
		Dose (ppm)	0	100	500	1500	0	100	500	1500
		No. of animals examined	19	16	17	17	16	19	18	19
Liver										
(liver cell)										
carcinoma			1	0	0	0	0	0	0	0
adenoma			1	0	2	0	1	0	0	1
clear cell foci	±		0	1	1	0	3	2	1	1
	+		3	3	3	4	1	2	1	2
basophilic foci	±		0	0	1	1	2	2	2	0
	+		4	4	4	2	3	3	2	3
vacuolated cell foci	±		0	1	0	1	0	1	1	0
	+		2	2	1	1	2	2	1	2
vacuolization	±		10	7	12	15	2	0	2	1
	+		4	1	0	0	3	0	0	5
	++		0	1	0	0	0	0	0	1
focal necrosis	±		3	1	3	3	5	3	0	2
	++		0	0	0	0	0	0	1	0
swelling	±		1	0	0	0	0	1	0	9
	+		0	0	1	0	0	0	0	1
cystic degeneration	±		2	3	1	0	0	0	0	0
microgranuloma	±		0	2	1	0	0	0	0	3
focal fibrosis	±		1	0	0	0	0	0	0	0
(Glisson's sheath)										
bile duct proliferation	±		9	1	2	3	9	13	13	8
	+		10	13	14	14	0	1	1	2
	++		0	2	1	0	0	0	0	0
cell infiltration	±		6	5	1	4	5	9	13	6
	+		0	0	0	0	0	0	0	2
(sinusoid)										
mononuclear cell increase	+		0	0	0	0	1	0	0	0
	++		1	1	0	0	0	0	0	1
Kidney										
(tubules)										
hyaline cast	±		9	4	2	2	10	14	7	7
	+		8	8	12	10	3	2	9	8
	++		2	3	3	5	0	0	2	3
	+++		0	0	0	0	0	0	0	1
dilatation	±		0	0	0	0	5	1	2	0
	+		1	0	0	0	0	1	3	2
	++		0	0	0	0	0	0	0	2
basophilic change	±		7	3	5	3	3	5	0	3
	+		10	11	11	11	0	0	0	1
	++		1	0	0	3	0	0	0	0
basement membrane thickening	±		12	7	3	1	7	7	11	8
	+		3	6	10	14	0	0	2	3
	++		0	3	3	2	0	0	0	1
brown pigment deposition	±		12	13	13	8	11	10	7	5
	+		3	1	2	4	1	1	2	4
calcification	±		0	0	0	0	2	3	4	3
dysplasia tubule	±		0	0	0	1	0	0	0	0
(glomerulus)										
Bowman's capsule thickening	±		10	9	3	6	1	1	1	0
	+		2	5	10	10	0	0	0	1
	++		0	0	2	0	0	0	0	0
(interstitium)										
cell infiltration	±		12	11	7	5	5	8	11	5
	+		2	4	10	12	0	0	1	4
	++		0	0	0	0	0	0	0	1
focal fibrosis	±		0	0	0	2	0	0	2	0
	+		1	3	3	0	0	0	0	1
	++		0	0	1	0	0	0	0	1
congestion	+		0	0	0	0	0	1	0	0
(pelvis)										
transitional epithelium hyperplasia	+		1	0	0	0	0	0	0	0
neutrophilic cell infiltration	+		1	0	0	0	0	0	0	0

±: slight, +: mild, ++: marked.

lar epithelium in the rat, also has an uncoupling effect on isolated liver mitochondria (Martinez, 1992) and sperm mitochondria (Breitbart *et al.*, 1989). Therefore, we speculated that the uncoupling action of MBEBP and MBMBP would play an important role in the observed testicular toxicity. In the present toxicity tests, suppression of body weight gain and testicular toxicity were not observed. The results suggest that IBBMP would not be an uncoupler on mitochondria. In the subchronic study, there were increased serum Na levels in males and increased Hb, MCH and MCHC levels in females. However, these effects were not observed in the chronic toxicity test. Although the reasons were not clear, adaptation may be induced in the chronic toxicity test. In chronic toxicity test, basophilic change, thickening of basement membrane in renal tubules were observed in 1500 ppm males at 6 months. At 18 months, dose-dependent increase in thickeness of basement membrane of renal tubules and Bowman's capsule and cell infiltration to the interstitium of kidney were observed in males. The mechanisms of nephrotoxicity are unknown. We have previously reported that MBEBP treatment for 18 months caused degenerated changes of kidney in male rats (Takagi *et al.*, 1996). In this study, increased tendency of eosinophilic body in renal tubules in the 1500 ppm males was observed at 6 months. Therefore, it is suggested that the accumulated eosinophilic bodies may play a role in the nephrotoxic effects. However, increased incidence of hyaline cast in females suggests that not only eosinophilic body accumulation but also other mechanisms would play an important role in the nephrotoxicity.

In conclusion, from the chronic toxicity test results, the no-observed-adverse-effect level (NOAEL) for IBBMP was estimated to be 100 ppm in the diet (4.26 mg/kg BW/day) in female rats on the basis of

Table 8. Statistical analysis of the histological findings in rats fed a diet containing IBBMP for 18 months.

Organs	Months	Sexes	Males				Females			
Findings		Dose (ppm)	0	100	500	1500	0	100	500	1500
Liver										
(liver cell)										
swelling	6		n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.
swelling	12		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
swelling	18		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
Kidney										
(tubules)										
basopilic change	6		n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.
hyaline cast	18		n.s.	n.s.	n.s.	*	n.s.	n.s.	*	*
basement membrane thickening	6		n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.
basement membrane thickening	18		n.s.	**	**	**	n.s.	n.s.	n.s.	n.s.
Bowman's capsule thickening	18		n.s.	n.s.	**	**	n.s.	n.s.	n.s.	n.s.
(interstitium)										
cell infiltration	18		n.s.	n.s.	**	**	n.s.	n.s.	*	n.s.

Statistical analysis was conducted by Fisher's exact probability test or Mann-Whitney U test: *p<0.05, **p<0.01, n.s.: not significant.

Table 9. Comparison of toxicity for IBBMP, MBEBP, MBMBP and BHT in rats.

	Hemorrhage	Testicular toxicity	Nephrotoxicity
IBBMP a	No	No	Yes
MBEBP b	No	Yes	Yes
MBMBP c	Yes	Yes	No
BHT d	Yes	No	No

a: Present study
b: Takagi A. *et al.* (1992), Takagi A. *et al.* (1996).
c: Takagi A. *et al.* (1994)
d: Takahashi O. *et al.* (1981), NTP (1979)

increased incidence of hyaline cast in renal tubules at 500 ppm. On the other hand, in males, only the lowest-observed-adverse-effect level (LOAEL) was given as 100 ppm (3.84 mg/kg BW/day) on the basis of induction of thickening of basement membrane in renal tubules at 100 ppm.

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Screening of Endocrine Disrupting Chemicals Using a Surface Plasmon Resonance Sensor

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Because concern over endocrine disrupting reactions caused by chemicals to humans and animals is growing, a rapid and reliable screening assay for endocrine disrupting chemicals is required. We have developed an *in vitro* screening assay based on a hormone receptor mechanism using a surface plasmon resonance (SPR) sensor. The interaction between an estrogen receptor α (ER) and an estrogen response element (ERE) is monitored in real time, when ER is injected over the SPR sensor chip on which a DNA fragment containing ERE is immobilized. In the presence of a chemical with estrogenic activity, the ER-ERE interaction is enhanced and the kinetic parameters are altered. We have validated the assay in terms of its specificity, dose dependency, optimal reaction conditions and reproducibility. It has been shown that the assay is very reliable as a rapid and quantitative screening method to judge the estrogenic activities of chemicals.

Introduction

Recently, concern has grown that some chemicals, such as organic chloride insecticides, plasticizers and detergents can cause endocrine disrupting effects to wild animals and humans.[1] Many of them are supposed to pose endocrine disrupting activities through direct interaction with the hormone receptors, such as estrogen receptor, thus modifying or inhibiting the physiological hormonal activities.[2] Chemical safety is evaluated by a set of the toxicological tests, such as carcinogenicity, teratogenicity, mutagenicity, reproduction tests. However, they have limitation to evaluate the chronic toxicity of chemicals. Moreover, the mechanisms of the endocrine disrupting activities are yet to be elucidated and the test methods to evaluate the effects are not well established.[3]

Several test methods have been reported to detect the endocrine disrupting activities caused by hormone receptors, *i.e.* competitive receptor binding assay, a cell growth assay using breast cancer cells expressing the estrogen receptors (MCF7),[4] cell-based reporter assay,[5] an *in vivo* rodent uterotrophic test,[6,7] and a vitellogenin assay using medaka fish (Oryzias latipes).[8] Many of these methods require a long time to obtain results. Furthermore, the endocrine disrupting activities can not always be detected when the chemicals are administered to the animals due to physiological regulations concerning the animal bodies. It is not easy to detect the hormonal effects of chemicals. The existing toxicological methods are not always the best way to detect the effects which have the feedback mechanism or the effects *via* the receptors. Therefore, a novel approach is sought for the rapid assessment of the endocrine disrupting activities of the chemicals.

The hormone receptors are the ligand dependent transcription factors. For example, estrogen receptor (ER) changes its conformation upon binding of the endogenous ligand, estrogen, and binds to the specific sequence of the DNA located upstream of the target genes and activates transcription of the genes (Fig. 1). Many chemicals with diverse structures have been reported to have estrogenic activities. Due to the variety of the structure, it is unlikely that all the chemicals act with the same mechanism. Each chemical may pose a different regulatory effect on the gene expression.[9]

Recently, a surface plasmon resonance (SPR) sensor is emerging as a novel analytical instrument.[10] The SPR sensor has features that it can monitor molecular interaction without labeling the molecules in real time. It is, therefore, suitable for high throughput screening assays. Compared to the existing technologies which monitor the binding amounts at the end of the interactions, the SPR sensor is unique to be able to detect the processes throughout the association and the dissociation of the interaction. This feature enables detailed analyses of chemical effects to receptors.

We have established a cell free screening assay focusing on the hormone receptor mechanism as a high throughput screening method for the endocrine disrupting chemicals. In order to measure the interaction of the biological molecules using the SPR sensor, one of the test molecules is immobilized

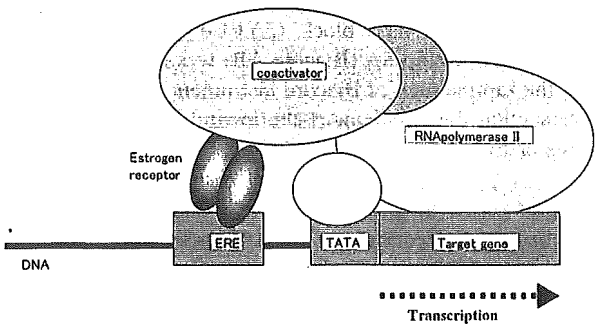

Fig. 1 Functional mechanism of the estrogen receptor in gene regulation.

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This is an English edition of the paper which won the Best Paper Award in Bunseki Kagaku, 2002 [*Bunseki Kagaku*, **2002**, *51(6)*, 389].

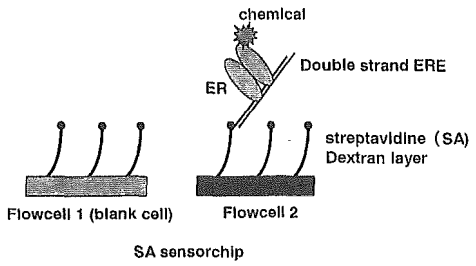


Fig. 2 Scheme of the ER-ERE assay with an SPR sensor.

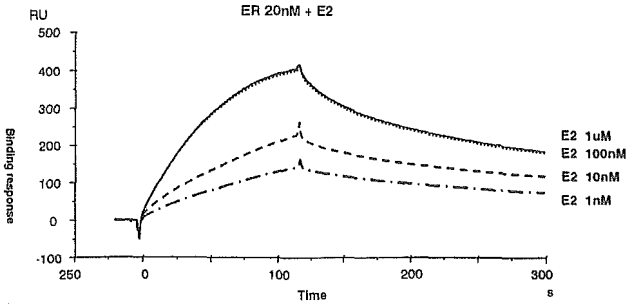


Fig. 3 Dose-dependent responses of E2.

on the sensor chip surface and a solution containing the other molecule is passed over the sensor surface at a constant flow rate through the microfluidics. A small mass changes, resulting from the binding and the dissociation of the two molecules on the sensor surface is monitored as SPR signals. The time course of the changes in the SPR signals is displayed as a curve called a sensorgram. Unlike from the conventional technologies, the SPR sensor can measure the interactions using a small amount of non-labeled samples within a short time. With regard to the interaction of the molecules, the SPR sensor can give not only the affinity of the two molecules at the equilibrium (as the dissociation constant, K_D or the affinity constant, K_A) but also the information on two molecules binding or dissociation velocity, namely the association rate constant (k_a) and the dissociation rate constant (k_d).

We have designed an assay to monitor the interaction of estrogen receptor α (ER) and the estrogen response element (ERE)[11] that is located in the promoter region of the estrogen target genes by immobilizing the DNA fragment containing the ERE sequence on the sensor chip and injecting purified ER over the sensor chip (Fig. 2). Thirty chemicals were tested for the estrogenic activities.

Experiments

Reagents and instruments

Reagent. Tricine, $CaCl_2$, $MgCl_2$, KOH, Tween 20, NaOH and HCl were purchased from Nacalai Tesque and DMSO from Sigma. Estrogen receptor (ER) was purchased from PanVera. ER was aliquoted into 5 μl and stored at –80°C. Biotinylated estrogen response element (ERE) DNA (5′-biotin-tcgagcaaagtcaggtcacagtgacctgatcaat-3′) of viterogenin gene and the anti-strand DNA have been synthesized by Nisshinbo. The synthesized oligomers were diluted with MilliQ water to 1 mg/ml and stored at –20°C. The running buffer for Biacore 3000 was prepared by filtering a solution of 25 mM Tricine, 160 mM KCl, 5 mM $MgCl_2$ (pH 7.8), 0.05% Tween 20.

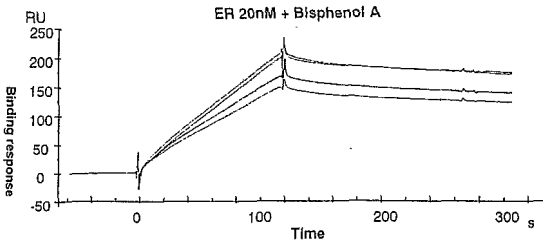
Instrument. The assay was performed using Biacore 3000 (Biacore AB), the heat block (EYELA) and the circulator (Asone). Sensor chip SA (Biacore AB) was used. Through the assay, the sample rack of Biacore instrument was cooled to 4°C by connecting the circulator to the instrument and the reaction was run at 25°C.

Operation

Immobilization of biotinylated ERE. For the immobilization of biotinylated ERE to the sensor chip, a streptoavidin preimmobilized sensor chip (Sensor chip SA) was set to the Biacore 3000 instrument and the instrument was equilibrated with running buffer. In order to stabilize the sensor surfaces, 100 mM NaOH and 50 mM HCl were injected for 30, 5 times. After checking the baseline stability, we performed immobilization of the biotinylated ERE. Biotinylated ERE (1 mg/ml) was diluted hundred thousand times with the running buffer. Then, 100 μl of the ERE solution was heated in boiling water for 5 min and chilled rapidly to denature the biotinylated ERE. The solution was injected over the sensor surface to immobilize approximately 60 RU onto the SA sensor chip surface. Then, the complementary ERE (1 mg/ml) was diluted to 100 times with the running buffer and denatured by the same method. This solution was injected for 2 min over the sensor surface where the biotinylated ERE was immobilized to form double stranded ERE on the surface. Biotin (1 μg/ml) was injected to block free SA on the sensor surface. A separate flow cell was used as a blank cell on which only biotin was immobilized.

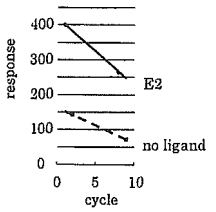
Preparation of the test chemicals. Each chemical was dissolved with 100% DMSO to make 0.1 M stock solution, and stored at –80°C. Immediately before the assay, 1 μl of the stock solution was diluted 500 times using the chilled running buffer. Also ER stock solution was diluted to 40 nM using a chilled running buffer. A 50-μl volume of the ER solution and 50 μl of the chemical solution of each concentration were mixed to give final concentrations of 20 nM ER and 10 μM to 1 nM of the chemical. The samples were kept at 4°C in a sample rack to maintain the ER activity. 17β-Estradiol was used as a positive control. First, we prepared the various concentration of 17β-estradiol (1 μM to 1 nM) and measured the binding of ER to ERE (Fig. 3). As the binding activity of ER to ERE was plataued over 100 nM 17β-estradiol, we decided to use 100 nM 17β-estradiol as a positive control in the following experiments. We also prepared a negative control solution which did not contain any chemicals. After the preparation of samples, the samples were treated at 37°C, 5 min and rapidly cooled. The samples were then set on the sample rack for measurements.

Assay of ER and ERE. The prepared samples were injected for 2 min at a flow rate of 20 μl/min over the immobilized ERE and the blank flowcell. Injection command of "kinject" was used and the dissociation phase was monitored for 2 min. The "kinject" command is one of the injection commands specially designed for the kinetic analysis in the Biacore instrument. Upon injecting the samples using "kinject" command, the sample solution was clearly separated by two air plugs at the both ends of the sample solution from the running buffer in order to prevent the sample solution from being diluted by the running buffer. The command is also designed to monitor dissociation of the bound molecule without being disturbed by the movement of the injection needle for the set period of time. After monitoring the binding and dissociation, 100 mM NaOH and 50 mM HCl were injected 30 s each for regeneration of the sensor surfaces. All the measurements were run automatically.



Cycle	analyte	conc nM	A	B	C
1	E2_1	100	400	400	151.48
2	no_ligand1	0	141.4	380.75	141.40
3	Bisphenol A	10,000	185.5	342.27	121.23
4	Bisphenol A	1,000	129	323.03	111.14
5	Bisphenol A	100	97.2	303.78	101.05
6	Bisphenol A	10	92.1	284.54	90.97
7	Bisphenol A	1	84.8	284.54	90.97
8	E2_2	100	265.3	265.30	80.88
9	no_lig2	0	70.8	246.05	70.80

A: Binding response with chemical
B: Calculated binding responses with 100nM E2
C: Calculated binding responses without chemical



$$\% \text{ activation} = \frac{A - C}{B - C} \times 100$$

Fig. 4 Data evaluation of chemical screening.

Data evaluation

A set of the assay consisted of 5 concentrations of the test chemical, a negative control (no chemical) and a positive control (100 nM 17β-estradiol). The results were compared as the ratio to the positive control (% activation). ER was unstable and lost its binding activity to ERE during the assay period in spite of optimizing the assay conditions. We have developed an assay design to correct for any loss of the binding activity of ER over time. A positive control cycle and a negative control cycle were run at the beginning and the end of the assay. The binding responses were recorded. Based on the rate of loss in the positive and negative control samples, the binding responses of the positive and negative controls for each cycle were calculated. The enhancement of ER binding by the test chemical was expressed as the ratio to those by the positive control of 100 nM 17β-estradiol, namely as a relative activation (% activation) using the formula and the corrected binding signals, as shown in Fig. 4.

Results

Validation of the ER-ERE assay using Biacore

In order to confirm the significance of the ER assay, the binding of ER to ERE was tested with a varying concentration of ER. The binding signals increased in relation to the increasing concentrations of ER. ER did not bind to the sensor surface where no ERE was immobilized (Fig. 5). A 1 μM volume of BSA did not show any significant binding to ERE surfaces (Fig. 6). These observations indicate that the assay monitors the specific binding of ER to ERE. Comparing the results with 10, 20 and 40 nM ER, we often observed relatively low binding signals with 10 nM ER. Higher binding signals were obtained by adding a final concentration of 1 μM BSA to 10 nM ER. Due to the low protein concentration, ER was lost by absorption to the surfaces of the plastic vials and tips and the actual concentration of ER became lower than 10 nM. Based on

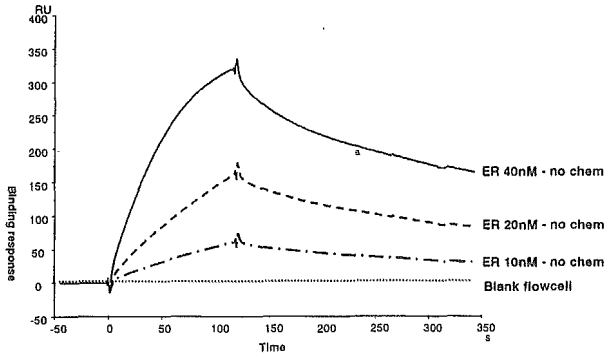


Fig. 5 Dose-dependent responses of ER-1.

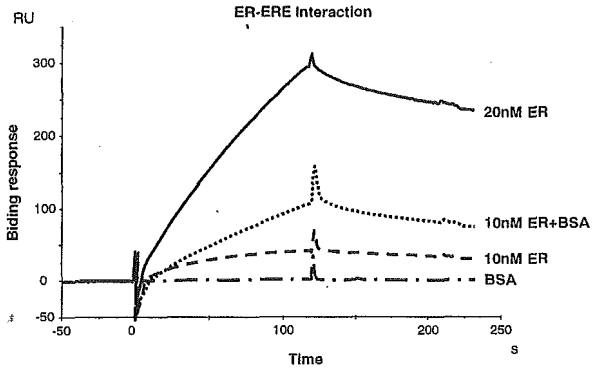


Fig. 6 Dose-dependent responses of ER-2.

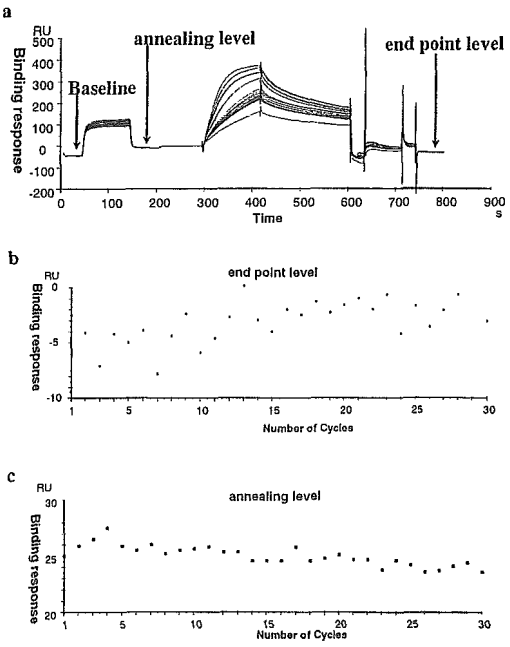


Fig. 7 Reproducibility of the ER-ERE assay.

those results, we decided to run the following assays with the final ER concentration of 20 nM.

It is important to regenerate the sensor surfaces to achieve reproducible results in the Biacore assay. We have repeated 30 cycles of the assay (Fig. 7a) and monitor the end point levels (Fig. 7b). It was confirmed that the sensor surfaces were properly regenerated and the assay showed high reproducibility.

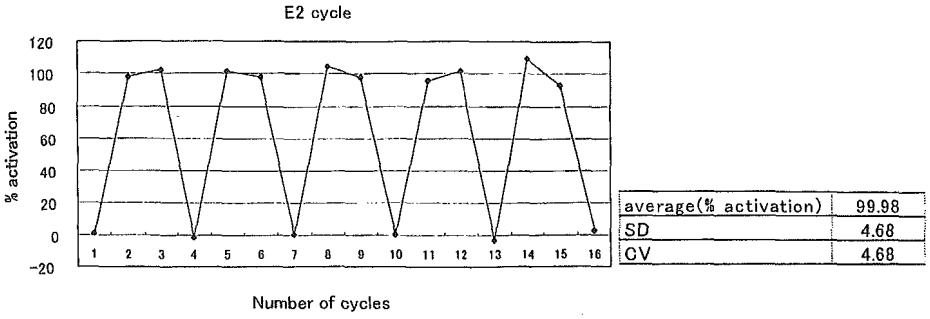


Fig. 8 Precision of the ER-ERE assay.

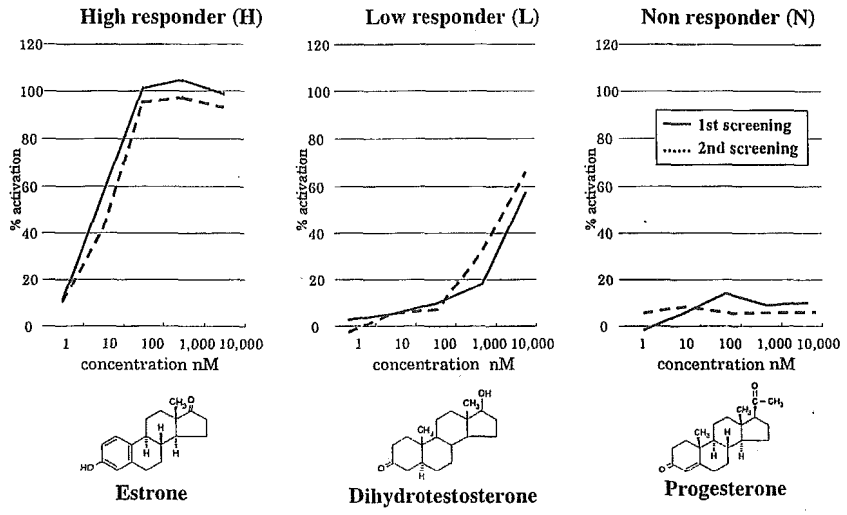


Fig. 9 Three types of chemical responses.

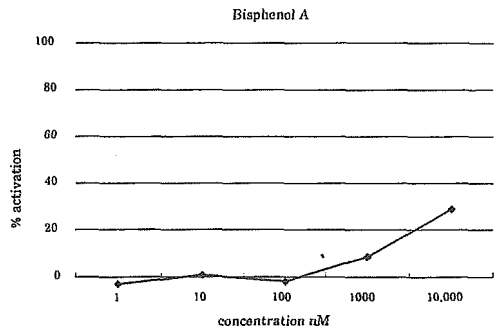


Fig. 10 Results of Bisphenol A.

The difference in the end point level was within 4 RU and the annealing level of anti-ERE was kept constant at around 25 RU throughout 30 cycles (Fig. 7c). We tested the reproducibility by repeating positive and negative controls for 16 cycles. It was shown that the results were with high precision with a CV% value of 4.68%, as shown in Fig. 8.

Screening results of 30 chemicals

We tested 30 chemicals to check the dose-dependent activation of the ER binding. One cycle of the assay took 15 min and the screening of one chemical was completed with 9 cycles in 2.5 h, including 5 different concentrations of the test chemicals, the positive and negative controls repeated twice for each control. We calculated the relative activation (% activation) using the formula shown in Fig. 4 for 30 chemicals. Based on the values of % activation at 100 nM of each chemical, chemicals could be classified into three groups (Fig. 9): the chemicals that showed more than 50% of the activation as "high responders", those with 20 - 50% as "low responders" and those less than 20% as "non-responders". The results obtained with two independent sets of screening were summarized in Table 1. 28 out of 30 chemicals showed the same results in the first and the second screening. 17β-Estradiol and its derivatives were classified to "high responders", while male hormones (progesterone) were "non-responders". Bisphenol A which is regarded as one of the endocrine disruptors, was classified among "low responders" (Fig. 10).

Furthermore, the differences in the effect of the chemicals on the ER binding activities were observed in the different shapes of the sensorgrams among those of 17β-estradiol, bisphenol A, 17α-estradiol, diethylstilbestrol (DES), tamoxifen and progesterone (Fig. 11). We have plotted the binding level at the end of the injection of ER in the presence of 1 μM of the test chemical (Y axis) *versus* the binding stability 2 min after the end of the ER injection (X axis), as shown in Fig. 12. We found that the agonists and the antagonists had significantly different patterns. The antagonists (such as tamoxifen) had a tendency to stabilize the binding of ER to ERE. The assay using Biacore indicated the possibility not only to detect the estrogenic activities of the chemicals, but to distinguish the antagonists from the agonists.

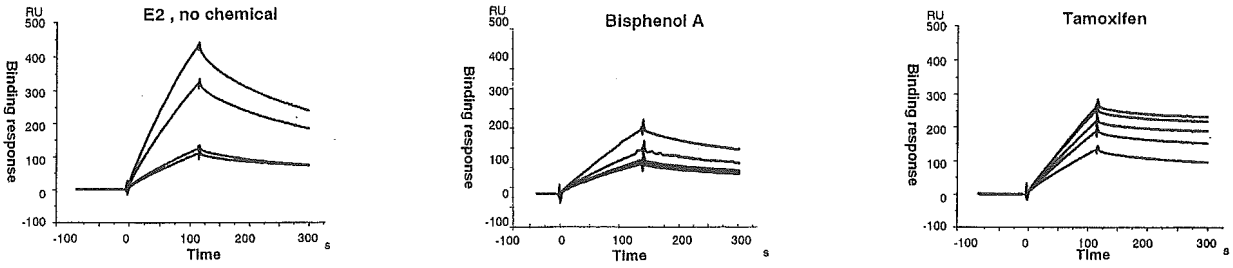


Fig. 11 Different kinetic patterns in the ER-ERE interaction.

Table 1 Results of ER-ERE screening with SPR sensor

No.	CAS No.	Name	1st Screening	2nd Screening
1	000050-28-2	Estradiol	H	H
2	000057-91-0	Estra-1,3,5(10)-triene-3,17-diol (17α)	H	H
3	000053-16-7	Estrone	H	H
4	000057-63-6	19-Nor-17-alpha-pregna-1,3,5(10)-trien-20-yne-3,17-diol	H	H
5	000362-05-0	Estra-1,3,5(10)-triene-2,3,17-beta-triol	H	H
6	000362-07-2	Estra-1,3,5(10)-triene-3,17-diol, 2-methoxy-, (17β)-	L	L
7	000068-22-4	19-Nor-17-alpha-pregn-4-en-20-yn-3-one, 17-hydroxy-	L	L
8	000063-05-8	Androst-4-ene-3,17-dione	N	N
9	000057-83-0	Progesterone	N	N
10	000501-24-6	3-Pentadecylphenol	N	N
11	005153-25-3	Benzoic acid, 4-hydroxy-, 2-ethylhexyl ester	N	L
12	001034-01-1	Gallic acid, octyl ester	N	N
13	006807-17-6	4,4'-(1,3-Dimethylbutylidene)bisphenol	L	L
14	027955-94-8	Phenol, 4,4',4"-ethylidynetri-	N	N
15	000081-92-5	Benzenemethanol, 2-[bis(4-hydroxyphenyl)methyl]-	L	L
16	000081-90-3	o-Toluic acid, .alpha.,.alpha.-bis(p-hydroxyphenyl)-	N	N
17	000978-86-9	4-(Triphenylmethyl)phenol	L	L
18	062625-31-4	Phenol, 4,4'-(3H-1,2-benzoxathiol-3-ylidene)bis 3-methyl-, S,S-dioxide, monosod	N	N
19	005384-21-4	Phenol, 4,4'-methylenebis[2,6-dimethyl-	L	L
20	005613-46-7	2,6-Xylenol, 4,4'-isopropylidenedi-	L	L
21	000084-16-2	Phenol, 4,4'-(1,2-diethylethylene)di-, meso-	H	H
22	000084-17-3	Phenol, 4,4'-(diethylideneethylene)di-	L	H
23	56-53-1	diethylstilbestrol	H	H
24	006893-02-3	Alanine, 3-(4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl)-, L-	N	N
25	000500-38-9	Nordihydroguaiaretic acid	N	N
26	023239-51-2	Benzyl alcohol, p-hydroxy-alpha-(1-((p-hydroxyphenethyl)amino)ethyl)-, hydrochlo	N	N
27	001050-28-8	L-Tyrosine, N-L-tyrosyl-	N	N
28	000145-50-6	1(4H)-Naphthalenone, 4- .alpha.-(4-hydroxy-1-naphthyl)benzylidene -	L	L
29	000446-72-0	Genistein	L	L
30	000080-05-7	Bisphenol A	L	L

H, High responder; L, low responder; N, non-responder.

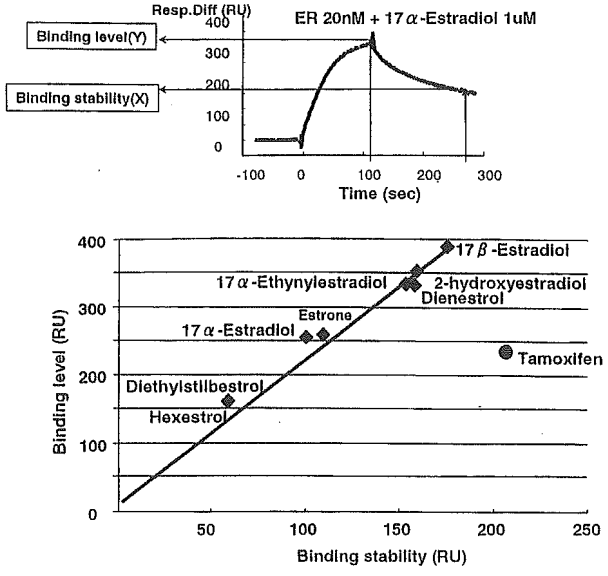


Fig. 12 Binding level *vs.* binding stability plot.

Discussion

We established a cell free screening method while focusing on the mechanism of the hormone receptor using a surface plasmon resonance sensor. We developed an assay method to detect estrogenic activities of the chemicals with changes in the binding level of ER to ERE by preincubating the chemicals with ER.

It was also suggested that the agonitst and the antagonists had different effects on the interaction of ER and ERE from an analysis of the binding level of ER during the association and dissociation processes. With the conventional end point assay used to monitor only the binding signals, it was impossible to distinguish the agonists from the antagonists. The real time analysis, which is the main feature of the surface plasmon resonance sensor enabled the classification of the agonists and the antagonists. When running the cell based hormone assay, it must be taken into account any unexpected effects of the chemicals to the other components than the receptors of the cells. On the other hand, the cell free assays simply show the