BPB Reports

Regular Article

Screening of Industrial and Agricultural Chemicals for Searching a Mouse PXR Activator Using Cell-Based Reporter Gene Assays

Ryota Shizu,[a] Makoto Kano,[a] Taiki Abe,[a,b] Saki Tsuchiya,[a] Yuki Shimizu,[a] Michiko Watanabe,[a] Takuomi Hosaka,[a] Takamitsu Sasaki,[a] and Kouichi Yoshinari[*,a,b]

[a]*Laboratory of Molecular Toxicology, School of Pharmaceutical Sciences, University of Shizuoka, 52–1 Yada, Suruga-ku, Shizuoka 422–8526 Japan;* [b]*Laboratory of Health Chemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, 6–3 Aramaki-Aoba, Aoba-ku, Sendai 980–8578 Japan*

Received September 24, 2018; Accepted September 25, 2018

The nuclear receptor pregnane X receptor (PXR, NR1I2) regulates several liver functions such as xenobiotic metabolism, energy metabolism, inflammation or cell growth, which are associated with drug-drug interactions and some diseases. It is well known that there are large species differences between human PXR and mouse PXR (mPXR) ligands. Although mouse models are often used in biological research, the number of mPXR ligands are limited. In the present study, we have thus searched mPXR activators from 190 industrial chemicals and 161 agricultural chemicals by reporter assay system with a promoter region of PXR target gene, and mouse primary hepatocytes and mice were treated with the candidates to confirm mPXR activation. Thirty-eight chemicals were selected after reporter assay screening. Among them, seven chemicals were selected as potential mPXR activators since their treatment increased mRNA levels of *Cyp3a11*, a representative PXR target gene, in mouse primary hepatocytes. Finally, in *in vivo* experiments using mice, hepatic *Cyp3a11* mRNA levels were induced by treatment with flusilazole and metconazole. These results suggest that these two chemicals function as mPXR activators *in vitro* and *in vivo*.

Key words nuclear receptor, *in vitro* screening, cytochrome P450 induction

INTRODUCTION

Pregnane X receptor (PXR, NR1I2) is a nuclear receptor highly expressed in the liver and intestine. The receptor is activated by the binding of its ligand xenobiotics and transactivates multiple genes encoding drug-metabolizing enzymes and drug transporters, which play key roles in xenobiotic disposition. In the basal condition, PXR is inactive and retained in the cytoplasm. Upon ligand binding, it is translocated into nucleus and forms a heterodimer with retinoid X receptor α (RXRα). The heterodimer binds to promoter regions of the target genes to induce their transcriptions. By inducing metabolism and excretion, PXR protects our body from harmful xenobiotics. In addition, ligand-activated PXR plays several roles in the liver or intestine such as energy metabolism,[1] cell proliferation,[2,3] cell migration[4,5] and inflammatory responses.[6,7]

For these studies, mice have been the leading models, and especially PXR-deficient and disease model mice are essential to these biomedical researches. However, there is a large species difference in PXR ligands between human and mouse, and mouse PXR (mPXR) ligands is limited while a lot of human PXR (hPXR) ligands have been identified including rifampicin, rifaximin, statins, clotrimazole, hyperforin and SR12813. The typical hPXR ligand rifampicin is unable to activate mPXR, and the typical mPXR activator pregnenolone 16α-carbonitrile (PCN) is inactive for hPXR.[8] Therefore, most of mouse studies on PXR utilizes PCN and basically the results have not been confirmed with other mPXR activators because of its unavailability. These facts indicate that it is of great importance to identify a mPXR activator(s) other than PCN.

In the present study, we have screened 190 industrial chemicals and 161 agricultural chemicals for their mPXR-activating abilities by reporter assay system with a promoter region of PXR target gene. Moreover, mouse primary hepatocytes and mice were treated with potential mPXR activators to investigate their effects in mouse livers.

MATERIALS AND METHODS

Reagents PCN was purchased from Sigma-Aldrich (St. Louis, MO). 5x(dNR1)-5x(eNR3A4)-pGL3 and mPXR-pTargeT were prepared previously.[9] phRL-TK (Promega, Madison, WI) was used as a control plasmid to normalize transfection efficacy.

Reporter Assay HepG2 cells (RIKEN BioResource Center, Tsukuba, Japan) were seeded on 96-well plates at 10,000 cells/well. The cells were transfected with 5x(dNR1)-5x(eNR3A4)-pGL3, mPXR-pTargeT and phRL-TK with Jet-PEI (Polyplus transfection, Illkirch, France). Twenty-four hours after transfection, cells were treated with test chemicals, 10 μM PCN or vehicle (final concentration of 0.1% dimethyl sulfoxide (DMSO)) for 24 h. The cell lysates were subjected to Dual Luciferase Assay System (Promega). Firefly luciferase

*To whom correspondence should be addressed. e-mail: yoshinari@u-shizuoka-ken.ac.jp

activity was normalized to *Renilla* luciferase activity.

Mouse Primary Hepatocyte Mouse primary hepatocytes were prepared and cultured as previously reported.[10] Cells were treated with test chemicals, 10 μM PCN or vehicle (final concentration of 0.1% DMSO) for 24 h and total RNA was extracted.

Animal Treatment All experiments were performed in accordance with the guidelines for animal experiments of University of Shizuoka. Seven to eight weeks old male C57BL/6N mice, (Charles River Japan, Yokohama, Japan) maintained in a temperature- and light-controlled environment (24°C, 12-h light/dark cycle), were intraperitoneally treated with test chemicals (100 mg/kg), PCN (100 mg/kg) or vehicle (corn oil, 20 mL/kg). Twenty-four hours after the treatment, mice were sacrificed by cervical dislocation and the livers were collected.

Determination of mRNA Levels Total RNA isolation and cDNA synthesis were carried out as described previously.[2] Quantitative reverse transcription-PCR (qRT-PCR) was performed using GoTaq qPCR Master Mix (Promega) and primer pairs for genes of interest as shown previously.[9] Target mRNA levels were normalized by *Actb* mRNA levels.

Statistical Analysis Statistical analysis was performed using JMP Pro 12 (SAS Institute, Cary, NC). All data are provided as the means ± SD. The significance of difference between control and treated groups was assessed using ANOVA followed by Dunnett's test.

RESULTS

We screened 190 industrial chemicals (Table 1) and 161 agricultural chemicals (Table 2) for their abilities to activate mPXR. The treatment with the typical mPXR ligand PCN, a positive control, induced reporter activities around 6-fold against the vehicle-treated group. Tables 1 and 2 show the relative reporter activities to those in PCN-treated cells, which were set at 100%. Among the test compounds, 17 industrial chemicals and 21 agricultural chemicals that greatly induced reporter activities and did not show obvious toxicities were selected for next screening as mPXR activator candidates.

Next, mouse primary hepatocytes were treated with the selected candidates for 24 h, and the mRNA levels of *Cyp3a11*, a PXR target gene, and *Cyp1a2*, a target gene of xenobiotic-responsive transcription factor aryl hydrocarbon receptor (AHR), as a negative control, were determined. As results, I33, I58, I74, I169, I174, A100, A124, A131 and A152 significantly increased *Cyp3a11* mRNA levels as did PCN (Fig. 1). I58, I117, I169, I190, A5, A40 and A152 also increased *Cyp1a2* mRNA levels with statistical significances (Fig. 1). According to the results, I33, I74, I174, A100, A124, A131 and A152 were selected for the 3rd screening.

In the 3rd screening, male mice were intraperitoneally treated with 100 mg/kg of each candidate, PCN or vehicle for 24 h, and hepatic RNAs were subjected to qRT-PCR to determine the mRNA levels of *Cyp3a11*, *Cyp1a2* and *Cyp2b10*, another PXR target gene. The results are shown in Fig. 2. A100 and A131 significantly increased *Cyp3a11* mRNA levels as much as PCN and increased *Cyp2b10* mRNA levels greater than PCN. A100 but not A131 slightly increased *Cyp1a2* mRNA levels.

In contrast, I33, I74, A124 had no effect on these mRNA levels except a minimal increase in *Cyp1a2* mRNA levels by A124. I174 and A152 slightly increased *Cyp3a11* mRNA levels, and strongly induced *Cyp1a2* mRNA levels, suggesting that they are AHR activators. From these results, agricultural chemicals A100 and A131 were identified as mPXR activators that work *in vivo*.

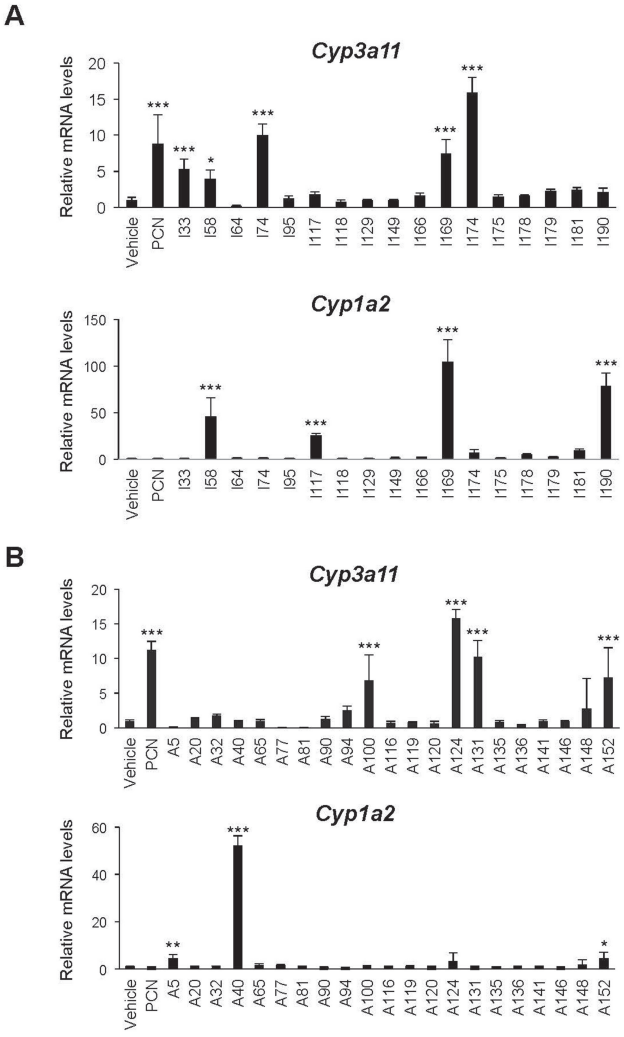

Fig. 1. Screening for mPXR Activators in Mouse Primary Hepatocytes

Mouse primary hepatocytes were treated with vehicle (0.1% DMSO), PCN (10 μM), or each industrial (100 μM; **A**) and agricultural chemicals (10 μM; **B**) for 24 h. Total RNA was extracted and subjected to qRT-PCR for *Cyp3a11* and *Cyp1a2*. Values are the mean ± SD (n = 4). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Dunnett's test versus vehicle-treated group).

DISCUSSION

In this study, we searched a mPXR activator(s) from industrial and agricultural chemicals and characterized two agricultural chemicals flusilazole (A100) and metconazole (A131) as potential mPXR activators (Fig. 3). We also identified several chemicals that activated mPXR in cell-based reporter assays and mouse primary hepatocytes but not in the liver of mice.

PXR sometimes shares its ligand with a very close nuclear receptor constitutive active/androstane receptor (CAR, NR1I3).[11] *Cyp3a11* and *Cyp2b10* are common target genes of mPXR and mouse CAR (mCAR), and increases in *Cyp3a11* and *Cyp2b10* mRNA levels in mouse livers are mainly regulated by mPXR and mCAR, respectively. Thus, by comparing the induction levels of these mRNA levels, we can assume which receptor is activated. Because flusilazole and metconazole

Table 1. List of Industrial Chemicals Tested and Their Induction Ratios in Reporter Assays

No.	CAS No.	Name	MW	% induction
I169	608-93-5	pentachlorobenzene	250.3	184.5
I58	84-51-5	2-ethylanthraquinone	236.3	170.2
I178	2440-22-4	2-(2*H*-benzotriazol-2-yl)-4-methyl-phenol	225.2	108.5
I117	82-45-1	1-aminoanthraquinone	223.2	73.9
I190	42240-73-3	2,2',3,3'-tetrachloro-4,4'-diaminodiphenylmethane	336.0	64.8
I166	6362-80-7	2,4-diphenyl-4-methyl-1-pentene	236.4	49.4
I129	95-73-8	2,4-dichlorotoluene	161.0	34.7
I95	620-92-8	4,4'-methylenediphenol	200.2	33.1
I175	80-04-6	hydrogenated bisphenol A	240.4	31.9
I149	118-79-6	2,4,6-tribromophenol	330.8	31.3
I33	78-51-3	tris(2-butoxyethyl) phosphate	398.5	28.0
I174	80-07-9	bis(*p*-chlorophenyl) sulfone	287.2	25.2
I179	2219-82-1	6-*tert*-butyl-*o*-cresol	164.2	24.9
I74	76-83-5	trityl chloride	278.8	23.5
I181	2173-57-1	2-isobutoxynaphthalene	200.3	22.8
I118	85-41-6	phthalimide	147.1	21.8
I64	102-06-7	1,3-diphenylguanidine	211.3	20.3
I42	1241-94-7	2-ethylhexyl diphenyl phosphate	362.4	17.9
I54	2416-94-6	2,3,6-trimethylphenol	136.2	17.9
I104	517-23-7	3-acetotetrahydrofuran-2-one	128.1	17.8
I188	208-96-8	acenaphthylene	152.2	17.5
I49	79-39-0	methacrylamide	85.1	17.4
I32	3648-21-3	diheptyl phthalate	362.5	16.7
I93	1025-15-6	1,3,5-tris(2-propenyl)isocyanuric acid	249.3	16.1
I105	2580-78-1	reactive blue 19	626.5	15.8
I186	91-96-3	*N*,*N*'-(3,3'-dimethylbiphenyl-4,4'-ylene)di(acetoacetamide)	380.4	15.7
I170	95-94-3	1,2,4,5-tetrachlorobenzene	215.9	14.7
I154	536-90-3	3-methoxybenzenamine	123.2	14.2
I122	89-72-5	*o*-sec-butylphenol	150.2	14.0
I12	99-71-8	4-(1-methylpropyl)phenol	150.2	13.8
I51	88-19-7	*o*-toluenesulfonamide	171.2	12.4
I176	99-54-7	1,2-dichloro-4-nitrobenzene	192.0	12.4
I59	88-18-6	2-*tert*-butylphenol	150.2	12.1
I165	4457-71-0	3-methyl-1,5-pentanediol	118.2	11.8
I127	92-88-6	4,4'-biphenyldiol	186.2	11.2
I86	95-32-9	2-(4-morpholinyldithio)benzothiazole	284.4	11.1
I83	7803-57-8	hydrazine monohydrate	50.1	10.8
I39	103-83-3	*N*,*N*-dimethylbenzylamine	135.2	10.4
I87	97-39-2	*N*,*N*'-bis(2-methylphenyl)guanidine	358.4	10.2
I121	88-60-8	6-*tert*-butyl-*m*-cresol	164.2	10.2
I1	95-64-7	3,4-dimethylaniline	121.2	9.7
I103	134-62-3	*N*,*N*-diethyl-*m*-toluamide	191.3	9.5
I180	103-44-6	3-[(vinyloxy)methyl]heptane	156.3	8.7
I145	111-82-0	methyl dodecanoate	214.3	8.5
I23	96-29-7	ethyl methyl ketoxime	87.1	8.3
I36	97-52-9	4-nitro-*o*-anisidine	168.2	7.9
I136	102-76-1	triacetin	218.2	7.7
I159	688-84-6	2-ethylhexyl methacrylate	198.3	7.5
I160	839-90-7	1,3,5-tris(2-hydroxyethyl)-1,3,5-triazine-2,4,6-(1*H,3H,5H*)-trione	261.2	7.4
I167	6846-50-0	2,2,4-trimethyl-1,3-pentanediol diisobutyrate	286.4	7.4
I123	89-83-8	thymol	150.2	7.3
I114	78-67-1	2,2'-azobis(2-methylpropanenitrile)	164.2	7.0
I13	106-37-6	1,4-dibromobenzene	235.9	6.6
I73	26471-62-5	tolylene diisocyanate	174.2	6.5
I140	107-66-4	dibutyl phosphate	210.2	6.2
I109	95-68-1	2,4-dimethylaniline	121.2	6.1
I172	125-33-7	primidone	218.3	6.0
I120	88-09-5	2-ethylbutanoic acid	116.2	5.9
I56	51-28-5	2,4-dinitrophenol	184.1	5.8
I142	108-65-6	1-methoxy-2-propanol acetate	132.2	5.8
I134	99-96-7	4-hydroxybenzoic acid	138.1	5.7
I189	123-63-7	2,4,6-trimethyl-1,3,5-trioxane	132.2	5.6
I162	3048-65-5	3a,4,7,7a-tetrahydro-1*H*-indene	120.2	5.2
I147	115-77-5	pentaerythritol	136.2	5.1
I161	882-33-7	diphenyl disulfide	218.3	5.1
I150	123-11-5	4-methoxybenzaldehyde	136.2	4.9
I45	127-68-4	sodium 3-nitrobenzenesulfonate	225.2	4.7
I11	86-87-3	1-naphthylacetic acid	186.2	4.5
I116	81-16-3	2-amino-1-naphthalenesulfonic acid	223.3	4.3
I81	109-64-8	1,3-dibromopropane	201.9	4.2
I139	105-45-3	methyl acetoacetate	116.1	4.2
I46	130-13-2	sodium 4-amino-1-naphthalenesulfonate	317.3	4.1
I50	80-09-1	bis(4-hydroxyphenyl)sulfone	250.3	4.1

Table 1. Continued

No.	CAS No.	Name	MW	% induction
I85	80-51-3	4,4'-oxybis(benzenesulfonyl hydrazide)	358.4	4.0
I107	29836-26-8	1-*O*-octyl -β-D-glucopyranoside	292.4	4.0
I187	103-64-0	β-bromostyrene	183.1	4.0
I152	126-30-7	2,2-dimethyl-1,3-propanediol	104.2	3.8
I20	87-02-5	7-amino-4-hydroxy-2-naphthalenesulfonic acid	239.3	3.7
I183	75-66-1	2-methylpropane-2-thiol	90.2	3.6
I71	620-17-7	*m*-ethylphenol	122.2	3.5
I157	611-19-8	1-chloro-2-(chloromethyl)benzene	161.0	3.5
I5	99-09-2	3-nitroaniline	138.1	3.4
I28	585-07-9	*tert*-butyl methacrylate	142.2	3.4
I84	56539-66-3	3-methoxy-3-methyl-1-butanol	118.2	3.2
I111	77-73-6	dicyclopentadiene	132.2	2.9
I48	16219-75-3	5-ethylidene-2-norbornene	120.2	2.6
I38	100-69-6	2-vinylpyridine	105.1	2.5
I153	512-56-1	trimethyl phosphate	140.1	2.5
I168	7580-85-0	2-*tert*-butoxyethanol	118.2	2.3
I171	51-79-6	urethane	89.1	2.3
I177	873-32-5	2-chlorobenzonitrile	137.6	2.1
I141	108-44-1	*m*-toluidine	107.2	2.0
I164	4189-44-0	thiourea *S*,*S*-dioxide	108.1	1.9
I99	100-47-0	benzonitrile	103.1	1.8
I119	87-62-7	2,6-dimethylaniline	121.2	1.8
I6	156-43-4	4-ethoxybenzenamine	137.2	1.5
I44	101-83-7	dicyclohexylamine	181.3	1.5
I100	108-87-2	methylcyclohexane	98.2	1.5
I35	87-59-2	2,3-xylidine	121.2	1.4
I62	95-57-8	2-chlorophenol	128.6	1.1
I184	57-30-7	phenobarbital sodium	255.2	0.8
I110	70-55-3	4-methylbenzenesulfonamide	171.2	0.7
I158	623-91-6	diethyl fumarate	172.2	0.7
I94	102-81-8	2-(di-*n*-butylamino)ethanol	173.3	0.6
I126	91-76-9	2,4-diamino-6-phenyl-1,3,5-triazine	187.2	0.5
I151	123-42-2	diacetone alcohol	116.2	0.5
I60	88-89-1	2,4,6-trinitrophenol	229.1	-0.1
I21	88-44-8	2-amino-5-methylbenzenesulfonic acid	187.2	-0.2
I113	77-99-6	2-ethyl-2-(hydroxymethyl)-1,3-propanediol	134.2	-0.2
I182	4130-42-1	2,6-di-*tert*-butyl-4-ethylphenol	234.4	-0.3
I37	100-54-9	3-cyanopyridine	104.1	-0.4
I75	98-51-1	*p*-*tert*-butyltoluene	148.3	-0.7
I65	106-48-9	4-chlorophenol	128.6	-0.9
I156	611-06-3	2,4-dichloro-1-nitrobenzene	192.0	-1.1
I43	3586-14-9	3-phenoxytoluene	184.2	-1.2
I31	2216-69-5	1-methoxynaphthalene	158.2	-1.4
I47	842-18-2	potassium 7-hydroxy-1,3-naphthalenedisulfonate	380.5	-1.4
I52	121-45-9	trimethoxyphosphine	124.1	-1.5
I55	5707-44-8	4-ethyl-1,1'-biphenyl	182.3	-1.6
I19	56-93-9	benzyltrimethylammonium chloride	185.7	-1.7
I2	100-61-8	*N*-methylaniline	107.2	-1.8
I115	78-97-7	2-hydroxypropanenitrile	71.1	-2.3
I34	83-32-9	acenaphthene	154.2	-2.6
I108	118-91-2	2-chlorobenzoic acid	156.6	-2.6
I66	108-39-4	3-methylphenol	108.1	-2.7
I146	111-88-6	1-octanethiol	146.3	-3.0
I4	105-99-7	dibutyl adipate	258.4	-3.1
I72	657-84-1	sodium *p*-toluenesulfonate	194.2	-3.2
I27	126-33-0	tetrahydrothiophene-1,1-dioxide	120.2	-3.3
I91	111-17-1	3,3'-thiobispropionic acid	178.2	-3.3
I155	542-18-7	chlorocyclohexane	118.6	-3.3
I16	583-39-1	2-mercaptobenzimidazole	150.2	-3.5
I92	112-26-5	1,2-bis(2-chloroethoxy)ethane	187.1	-3.5
I78	79-27-6	tetrabromoethane	345.7	-3.9
I125	91-15-6	1,2-benzenedicarbonitrile	128.1	-4.3
I132	98-83-9	1-methylethenylbenzene	118.2	-4.3
I137	103-24-2	bis(2-ethylhexyl) nonanedioate	412.7	-4.3
I143	108-80-5	isocyanuric acid	129.1	-4.3
I69	591-27-5	3-aminophenol	109.1	-4.4
I40	108-69-0	3,5-dimethylaniline	121.2	-4.6
I29	626-17-5	1,3-dicyanobenzene	128.1	-4.9
I88	97-99-4	tetrahydrofurfuryl alcohol	102.1	-4.9
I112	77-85-0	1,1,1-tris(hydroxymethyl)ethane	120.2	-5.0
I96	96-45-7	2-imidazolidinethione	102.2	-5.1
I102	121-60-8	*p*-(acetylamino)benzenesulfonyl chloride	233.7	-5.6

Table 1. Continued

No.	CAS No.	Name	MW	% induction
I61	95-50-1	*o*-dichlorobenzene	147.0	-5.7
I98	96-49-1	1,3-dioxolan-2-one	88.1	-6.0
I130	97-88-1	butyl methacrylate	142.2	-6.0
I77	6099-57-6	1-naphthol-4-sulfonic acid sodium salt	246.2	-6.3
I22	95-63-6	1,2,4-trimethylbenzene	120.2	-6.7
I128	93-68-5	*o*-acetoacetotoluidide	191.2	-6.7
I3	103-69-5	*N*-ethylaniline	121.2	-6.8
I10	5460-9-3	monosodium 4-amino-5-hydroxy-2,7-naphthalenedisulfonate	341.3	-6.9
I148	118-69-4	2,6-dichlorotoluene	161.0	-7.0
I144	110-02-1	thiophene	84.1	-7.2
I90	100-74-3	4-ethylmorpholine	115.2	-7.4
I30	1843-05-6	2-hydroxy-4-(octyloxy)benzophenone	326.4	-7.5
I25	111-41-1	*N*-(aminoethyl)ethanolamine	104.2	-7.6
I131	98-08-8	trifluoromethylbenzene	146.1	-7.6
I89	99-94-5	4-methylbenzoic acid	136.2	-7.8
I7	88-53-9	2-amino-5-chloro-4-methyl-benzenesulfonic acid	221.7	-8.7
I133	99-04-7	3-methyl benzoic acid	136.2	-8.9
I14	121-47-1	3-aminobenzenesulfonic acid	173.2	-9.4
I15	526-78-3	2,3-dibromosuccinic acid	275.9	-9.6
I76	108-73-6	1,3,5-trihydroxybenzene	126.1	-9.6
I17	623-26-7	terephthalonitrile	128.1	-10.0
I18	1477-55-0	1,3-bis(aminomethyl)benzene	136.2	-11.6
I163	3452-97-9	3,5,5-trimethyl-1-hexanol	144.3	-12.1
I124	90-02-8	2-hydroxybenzaldehyde	122.1	-12.9
I82	1552-42-7	3,3-bis(*p*-dimethylaminophenyl)-6-dimethylaminophthalide	415.5	-18.1
I106	4435-53-4	3-methoxy-*n*-butylacetate	146.2	-18.3
I138	105-16-8	2-(diethylamino)ethyl methacrylate	185.3	-20.1
I67	109-70-6	1-bromo-3-chloropropane	157.4	-96.6
I8	140-66-9	*p*-*tert*-octylphenol	206.3	N/A
I9	538-75-0	*N*,*N*'-dicyclohexylcarbodiimide	206.3	N/A
I24	96-69-5	4,4'-thiobis(6-*tert*-butyl-*m*-cresol)	358.5	N/A
I26	119-47-1	2,2'-methylenebis(6-*tert*-butyl-*p*-cresol)	340.5	N/A
I41	123-30-8	4-aminophenol	109.1	N/A
I53	793-24-8	*N*-(1,3-dimethylbutyl)-*N*'-phenyl-*p*-phenylenediamine	268.4	N/A
I57	79-94-7	4,4'-isopropylidenebis(2,6-dibromophenol)	543.9	N/A
I63	96-76-4	2,4-di-*tert*-butylphenol	206.3	N/A
I68	123-07-9	4-ethylphenol	122.2	N/A
I70	599-64-4	*p*-(α,α-dimethylbenzyl)phenol	212.3	N/A
I79	101-72-4	*N*-phenyl-*N*'-isopropyl-*p*-phenylenediamine	226.3	N/A
I80	109-59-1	2-(1-methylethoxy)ethanol	104.2	N/A
I97	77-90-7	acetyl tributyl citrate	402.5	N/A
I101	118-75-2	2,3,5,6-tetrachloro-*p*-benzoquinone	245.9	N/A
I135	101-14-4	4,4'-methylenebis(2-chlorobenzenamine)	267.2	N/A
I173	87-86-5	pentachlorophenol	266.3	N/A
I185	84852-15-3	4-nonylphenol, branched	220.4	N/A

Reporter assays were performed as described in Materials and Methods. The values of % induction indicate the ratios (%) of reporter activities to those in the cells treated with PCN being set as 100%. The values are the means of quadruplicate. MW, molecular weight. N/A, reporter activities were not detected because of cell death.

Table 2. List of Agricultural Chemicals Tested and Their Induction Ratios in Reporter Assays

No.	CAS No.	Name	MW	% induction
A148	148477-71-8	spirodiclofen	411.3	478.3
A135	135590-91-9	mefenpyr-diethyl	373.2	102.3
A5	131860-33-8	azoxystrobin	403.4	90.3
A119	40487-42-1	pendimethalin	281.3	87.0
A40	122453-73-0	chlorfenapyr	407.6	84.6
A77	175013-18-0	pyraclostrobin	387.8	81.3
A120	110956-75-7	pentoxazone	353.8	79.9
A136	55814-41-0	mepronil	269.4	78.7
A152	119-12-0	pyridaphention	340.3	75.1
A65	141517-21-7	trifloxystrobin	408.4	73.6
A90	55-38-9	fenthion	278.3	64.3
A100	85509-19-9	flusilazole	315.4	58.7
A32	124495-18-7	quinoxyfen	308.1	58.1
A124	374726-62-2	mandipropamid	411.9	54.0
A81	96489-71-3	pyridaben	364.9	52.4
A20	153233-91-1	etoxazole	359.4	51.3
A94	36335-67-8	butamifos	332.4	50.4
A141	2597-03-7	phenthoate	320.4	49.2

Table 2. Continued

No.	CAS No.	Name	MW	% induction
A131	125116-23-6	metoconazole	319.8	46.1
A116	22781-23-3	bendiocarb	223.2	45.9
A146	1582-09-8	trifluralin	335.3	43.7
A91	158237-07-1	fentrazamide	349.8	43.0
A144	143390-89-0	kresoxim-methyl	313.3	41.5
A101	66332-96-5	flutolanil	323.3	38.9
A64	78-48-8	tribufos	347.3	38.8
A15	86598-92-7	imibenconazole	411.7	38.1
A85	95737-68-1	pyriproxyfen	321.4	37.2
A155	64249-01-0	anilofos	367.9	36.7
A145	22936-75-0	dimethametryn	255.4	35.8
A63	24017-47-8	triazophos	313.3	34.0
A110	74712-19-9	bromobutide	312.3	33.7
A74	179877-41-8	bifenazate	300.4	33.5
A118	183675-82-3	penthiopyrad	359.4	33.5
A25	153197-14-9	oxaziclomefone	376.3	33.0
A89	161326-34-7	fenamidone	311.4	32.8
A26	42874-03-3	oxyfluorfen	361.7	32.4
A154	97-17-6	dichlofenthion	315.2	32.2
A27	248593-16-0	orysastrobin	391.4	31.4
A88	115852-48-7	fenoxanil	329.2	31.2
A121	1861-40-1	benfluralin	355.3	28.1
A105	51218-49-6	pretilachlor	311.9	27.8
A99	131341-86-1	fludioxonil	248.2	27.6
A28	95465-99-9	cadusafos	270.4	27.3
A130	161050-58-4	methoxyfenozide	368.5	27.2
A47	97886-45-8	dithiopyr	401.4	27.0
A12	26087-47-8	iprobenfos	288.3	24.9
A108	122-42-9	propham	179.2	24.3
A83	135186-78-6	pyriftalid	318.4	23.6
A139	15972-60-8	alachlor	269.8	23.6
A160	119446-68-3	difenoconazole	406.3	23.6
A45	141-66-2	dicrotophos	237.2	23.2
A114	66063-05-6	pencycuron	328.8	23.1
A92	114369-43-6	fenbuconazole	336.8	22.4
A43	139920-32-4	diclocymet	313.2	21.4
A86	799247-52-2	pyribencarb	361.8	21.4
A123	188425-85-6	boscalid	343.2	20.5
A115	83055-99-6	bensulfuron methyl	410.4	19.6
A158	658066-35-4	flufenacet	396.7	19.5
A150	881685-58-1	isopyrazam	359.4	19.0
A93	126833-17-8	fenhexamid	302.3	18.3
A129	2032-65-7	methiocarb	225.3	17.2
A41	260121-52-0	cyenopyrafen	393.5	16.8
A2	86-50-0	azinphos-methyl	317.1	15.8
A39	2921-88-2	chlorpyrifos	350.6	15.8
A23	19666-30-9	oxadiazon	345.2	15.4
A24	39807-15-3	oxadiargyl	341.2	15.1
A82	179101-81-6	pyridalyl	491.1	15.0
A109	181274-15-7	propoxycarbazone-sodium	421.3	14.3
A137	103055-07-8	lufenuron	511.2	14.3
A151	20354-26-1	methazole	270.3	13.7
A17	85785-20-2	esprocarb	265.4	12.9
A133	51218-45-2	metolachlor	283.8	12.9
A37	84496-56-0	clomeprop	249.1	12.5
A16	83659-17-4	uniconazole P	291.8	12.2
A70	27314-13-2	norflurazon	303.7	12.0
A102	101463-69-8	flufenoxuron	488.5	11.7
A29	125306-83-4	cafenstrole	350.4	11.3
A75	82657-04-3	bifenthrin	422.9	11.3
A61	107534-96-3	Tebuconazole	307.8	11.1
A21	80884-07-1	etofenprox	376.5	11.0
A78	158353-15-2	pyraclonil	314.8	10.9
A97	229977-93-9	fluacrypyrim	426.4	10.1
A98	239110-15-7	fluopicolide	383.6	9.9
A50	180409-60-3	cyflufenamid	412.4	9.4
A103	272451-65-7	flubendiamide	682.4	9.4
A80	129630-19-9	pyraflufen-ethyl	413.2	8.9
A53	110488-70-5	dimethomorph	387.9	8.3
A11	50512-35-1	isoprothiolane	290.4	8.1
A127	10265-92-6	methamidophos	141.1	8.1
A134	73250-68-7	mefenacet	298.4	8.1

Table 2. Continued

No.	CAS No.	Name	MW	% induction
A46	37764-25-3	dichlormid	208.1	7.4
A107	158-474-72-7	prohydrojasmon	254.4	6.8
A126	108-62-3	metaldehyde	176.2	6.8
A149	98967-40-9	flumetsulam	325.3	6.8
A62	115410-23-8	tebufenozide	352.5	5.9
A161	121552-61-2	cyprodinil	225.3	5.9
A14	138261-41-3	imidacloprid	255.7	5.7
A3	135410-20-7	acetamiprid	222.7	5.4
A18	55283-68-6	ethalfluralin	333.3	5.3
A104	59756-60-4	fluridone	329.3	4.6
A54	105024-66-6	silafluofen	408.6	4.2
A73	137641-05-5	picolinafen	376.3	4.2
A4	30560-19-1	acephate	183.2	4.1
A79	365400-11-9	pyrasulfotole	362.3	4.0
A128	70630-17-0	metalaxyl	279.3	3.9
A19	181587-01-9	ethiprole	397.2	3.8
A132	133408-50-1	metominostrobin	284.3	3.3
A36	143807-66-3	chromafenozide	394.5	3.2
A106	158062-67-0	flonicamid	229.2	2.9
A59	18249-77-6	thiobencarb	257.8	2.6
A51	149508-90-7	simeconazole	293.4	2.4
A52	87674-68-8	dimethenamid	275.8	2.2
A13	104098-48-8	imazapic	275.3	2.1
A42	113136-77-9	cyclanilide	274.1	1.9
A72	100764-20-1	halosulfuron-methyl	434.8	1.5
A112	98730-04-2	benoxacor	260.1	1.5
A44	145701-21-9	diclosulam	405.0	1.2
A84	337458-27-2	pyrifluquinazon	464.3	1.1
A142	123572-88-3	furametpyr	333.8	1.1
A156	2212-67-1	molinate	187.3	1.0
A38	54593-83-8	chlorethoxyfos	336.0	0.7
A95	69327-76-0	buprofezin	305.4	0.7
A9	834-12-8	ametryn	227.3	0.5
A10	141112-29-0	isoxaflutole	359.3	-0.7
A33	99485-76-4	cumyluron	302.8	-0.8
A122	68505-69-1	benfuresate	256.3	-0.9
A58	153719-23-4	thiamethoxam	291.7	-1.2
A31	76578-14-8	quizalofop-ethyl	372.8	-1.3
A117	177406-68-7	benthiavalicarb-isopropyl	381.5	-1.3
A55	66215-27-8	cyromazine	166.2	-1.4
A125	88671-89-0	myclobutanil	288.8	-1.4
A113	219714-96-2	penoxsulam	483.4	-1.5
A96	208465-21-8	primisulfuron-methyl	468.3	-1.9
A49	165252-70-0	dinotefuran	202.2	-2.0
A1	62476-59-9	acifluorfen	361.7	-2.1
A8	61-82-5	amitrole	84.1	-2.5
A68	4684-94-0	nitrapyrin	290.9	-2.6
A22	13194-48-4	ethoprophos	242.3	-3.0
A60	51707-55-2	thidiazuron	220.3	-3.6
A111	51235-04-2	hexazinone	252.3	-4.2
A48	142891-20-1	cinidon-ethyl	394.3	-4.5
A153	1918-00-9	dicamba	221.0	-4.6
A7	33089-61-1	amitraz	293.4	-5.2
A87	221205-90-9	pyrimisulfan	419.4	-6.1
A30	5234-68-4	carboxin	235.3	-7.8
A34	77182-82-2	glufosinate-ammonium	198.2	-8.0
A67	86-87-3	1-naphthaleneacetic acid, sodium salt	186.2	-8.7
A35	210880-92-5	clothianidin	249.7	-9.5
A66	129558-76-5	tolfenpyrad	383.9	-10.3
A76	123312-89-0	pymetrozine	217.2	-15.1
A69	116714-46-6	novaluron	492.7	-16.5
A71	76738-62-0	paclobutrazol	293.8	-22.5
A159	142459-58-3	fluopyram	363.3	-59.4
A147	76674-21-0	flutriafol	301.3	-270.4
A6	348635-87-0	amisulbrom	466.3	N/A
A56	283594-90-1	spiromesifen	370.5	N/A
A57	156052-68-5	zoxamide	336.7	N/A
A138	2104-64-5	EPN	323.3	N/A
A140	23184-66-9	butachlor	311.9	N/A
A143	71751-41-2	abamectin	887.1	N/A
A157	1918-16-7	propachlor	211.7	N/A

The values were determined and are presented as in Table 1.

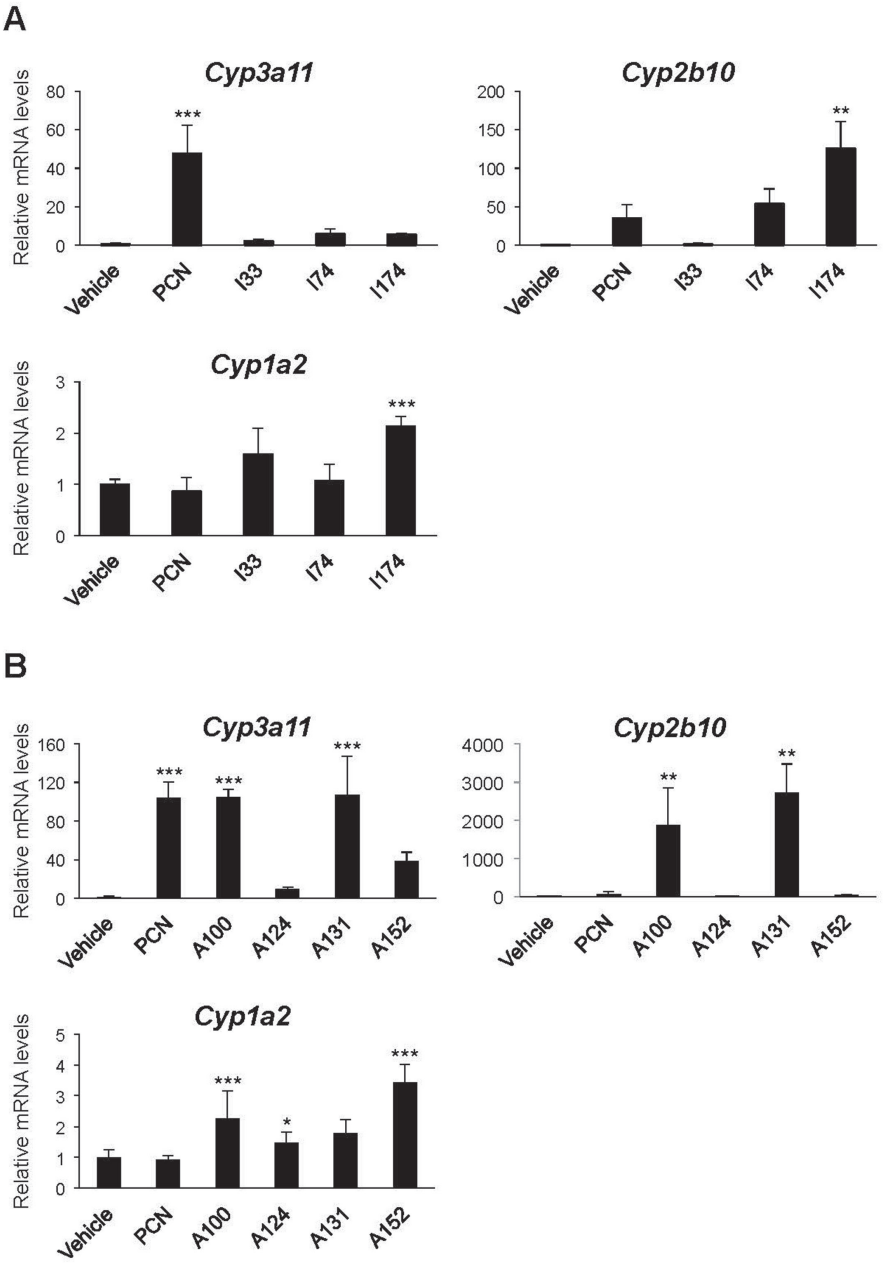

Fig. 2. Screening for mPXR Activators in Mice *In Vivo*

A, B. Male mice were treated intraperitoneally with vehicle (corn oil), PCN (100 mg/kg), or each test compound (100 mg/kg). Twenty-four hours later, total hepatic RNA was extracted and subjected to qRT-PCR for *Cyp3a11*, *Cyp2b10* and *Cyp1a2*. Values are the mean ± SD (n = 3-4). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Dunnett's test, versus vehicle-treated group).

induced *Cyp2b10* mRNA levels much more than PCN, they may activate not only mPXR but also mCAR. Both of them are azole-class antifungal agents. Azole-class antifungals and antibiotics are often reported as inducers of cytochrome P450s (P450s) *via* PXR and CAR activation[12] as well as P450 inhibitors. For example, clotrimazole, known as a hPXR activator, is also a CAR antagonist.[11] Voriconazole is considered as a dual agonist of mCAR and mPXR.[13] Although our results clearly indicate that flusilazole and metconazole are reliable P450 inducers at least in mice, it is still required to reveal whether they are specific agonist for mPXR.

Because the ligand-dependent PXR activation has been shown to be species specific, typical hPXR specific ligands such as rifampicin or SR12813 cannot be used as PXR activators in rodent studies. Thus, the studies are usually conducted with the mouse and rat PXR-specific ligand PCN, which is the only confirmed and reliable mPXR activator. However, PCN is reported to antagonize glucocorticoid receptor[14] and has PXR independent anti-inflammatory or anti-fibrogenesis effects.[15,16] Therefore, using only PCN as a mPXR activator may lead to misunderstandings of PXR's functions, and thus more mPXR activators are required. Some chemicals are reported as potential mPXR ligands such as 5β-pregnane-3,20-dione, amprenavir and imazalil,[8,9,17] although further studies are needed. In this study, we found two mPXR activating chemicals flusilazole and metconazole. These chemicals may help us to reveal the physiological, pathophysiological and toxicological functions of PXR by biomedical studies using mice.

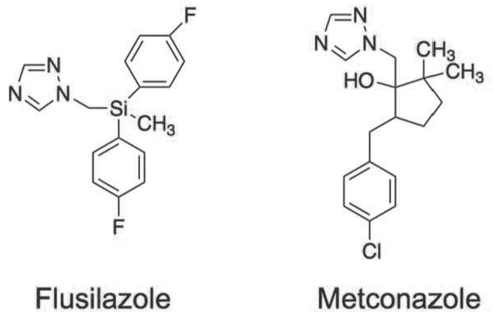

Fig. 3. Chemical Structures of mPXR Activators Identified

Acknowledgments This work was supported by JSPS KAKENHI Grant Number 17893283.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Hakkola J, Rysa J, Hukkanen J. Regulation of hepatic energy metabolism by the nuclear receptor PXR. *Biochim. Biophys. Acta,* **1859**, 1072–1082 (2016).
2) Shizu R, Benoki S, Numakura Y, Kodama S, Miyata M, Yamazoe Y, Yoshinari K. Xenobiotic-Induced Hepatocyte Proliferation Associated with Constitutive Active/Androstane Receptor (CAR) or Peroxisome Proliferator-Activated Receptor alpha (PPARalpha) Is Enhanced by Pregnane X Receptor (PXR) Activation in Mice. *PLoS One,* **8**, e61802 (2013).
3) Shizu R, Abe T, Benoki S, Takahashi M, Kodama S, Miayata M, Matsuzawa A, Yoshinari K. PXR stimulates growth factor-mediated hepatocyte proliferation by cross-talk with the FOXO transcription factor. *Biochem. J.,* **473**, 257–266 (2016).
4) Kodama S, Negishi M. Pregnane X receptor PXR activates the GADD45beta gene, eliciting the p38 MAPK signal and cell migration. *J. Biol. Chem.,* **286**, 3570–3578 (2011).
5) Kodama S, Yamazaki Y, Negishi M. Pregnane X Receptor Represses HNF4alpha Gene to Induce Insulin-Like Growth Factor-Binding Protein IGFBP1 that Alters Morphology of and Migrates HepG2 Cells. *Mol. Pharmacol.,* **88**, 746–757 (2015).
6) Mencarelli A, Renga B, Palladino G, Claudio D, Ricci P, Distrutti E, Barbanti M, Baldelli F, Fiorucci S. Inhibition of NF-kappaB by a PXR-dependent pathway mediates counter-regulatory activities of rifaximin on innate immunity in intestinal epithelial cells. *Eur. J. Pharmacol.,* **668**, 317–324 (2011).
7) Zhou C, Tabb MM, Nelson EL, Grun F, Verma S, Sadatrafiei A, Lin M, Mallick S, Forman BM, Thummel KE, Blumberg B. Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J. Clin. Invest.,* **116**, 2280–2289 (2006).
8) Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA, Moore JT. The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol. Endocrinol.,* **14**, 27–39 (2000).
9) Yoshimaru S, Shizu R, Tsuruta S, Amaike Y, Kano M, Hosaka T, Sasaki T, Yoshinari K. Acceleration of murine hepatocyte proliferation by imazalil through the activation of nuclear receptor PXR. *J. Toxicol. Sci.,* **43**, 443–450 (2018).
10) Abe T, Amaike Y, Shizu R, Takahashi M, Kano M, Hosaka T, Sasaki T, Kodama S, Matsuzawa A, Yoshinari K. Role of YAP activation in nuclear receptor CAR-mediated proliferation of mouse hepatocytes. *Toxicol. Sci.*, **165**, 408-419 (2018).
11) Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL, Kliewer SA. Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J. Biol. Chem.,* **275**, 15122–15127 (2000).
12) Goetz AK, Bao W, Ren H, Schmid JE, Tully DB, Wood C, Rockett JC, Narotsky MG, Sun G, Lambert GR, Thai SF, Wolf DC, Nesnow S, Dix DJ. Gene expression profiling in the liver of CD-1 mice to characterize the hepatotoxicity of triazole fungicides. *Toxicol. Appl. Pharmacol.,* **215**, 274–284 (2006).
13) Ohbuchi M, Yoshinari K, Kaneko H, Matsumoto S, Inoue A, Kawamura A, Usui T, Yamazoe Y. Coordinated roles of pregnane X receptor and constitutive androstane receptor in autoinduction of voriconazole metabolism in mice. *Antimicrob. Agents Chemother.,* **57**, 1332–1338 (2013).
14) Schuetz EG, Guzelian PS. Induction of cytochrome P-450 by glucocorticoids in rat liver. II. Evidence that glucocorticoids regulate induction of cytochrome P-450 by a nonclassical receptor mechanism. *J. Biol. Chem.,* **259**, 2007–2012 (1984).
15) Kodama S, Shimura T, Kuribayashi H, Abe T, Yoshinari K. Pregnenolone 16alpha-carbonitrile ameliorates concanavalin A-induced liver injury in mice independent of the nuclear receptor PXR activation. *Toxicol. Lett.,* **271**, 58–65 (2017).
16) Marek CJ, Tucker SJ, Konstantinou DK, Elrick LJ, Haefner D, Sigalas C, Murray GI, Goodwin B, Wright MC. Pregnenolone-16alpha-carbonitrile inhibits rodent liver fibrogenesis via PXR (pregnane X receptor)-dependent and PXR-independent mechanisms. *Biochem. J.,* **387**, 601–608 (2005).
17) Helsley RN, Sui Y, Ai N, Park SH, Welsh WJ, Zhou C. Pregnane X receptor mediates dyslipidemia induced by the HIV protease inhibitor amprenavir in mice. *Mol. Pharmacol.,* **83**, 1190–1199 (2013).