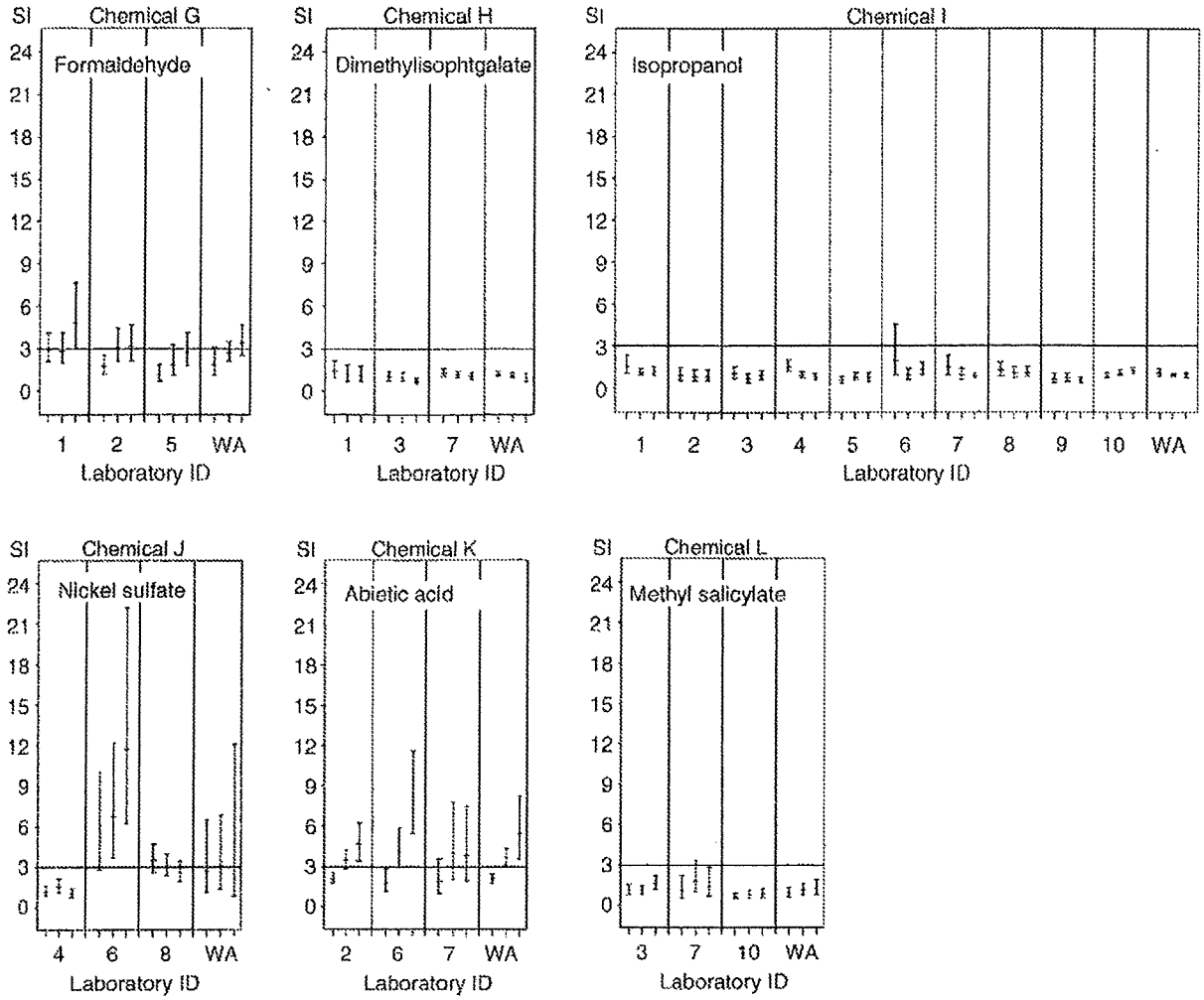

Fig. 1 (*continued*).

The GPMT/BT results for chemical D (glutaraldehyde) are not listed in Table 1(a) because the data were not available at the time the list was prepared.

The chemical selectors initially set the dose concentrations of chemical E (cobalt chloride) at 1%, 3%, and 10%. However, during the first round of the experiments in a laboratory in the first study, 2 of the 4 mice treated with the 10% dose concentration died, while the other 2 exhibited signs of hypokinesia. Since only the laboratory had conducted the experiment using this chemical concentration at the time, the chemical selectors decided to alter the dose concentrations. Then, the dose concentrations of chemical E were subsequently set at 0.3%, 1%, and 3% in a blinded manner for the remaining 2 laboratories in the first study. However, after several considerations, the chemical selectors adopted different doses in the second study, i.e., 1%, 3%, and 5%.

3.2. Body weights

Tables 2(a) and 2(b) summarize the body weight statistics observed on days 1 and 8 in each laboratory, respectively. No substantial interlaboratory variations were observed with regard to the body weights.

3.3. ATP content and SI values

The ATP content and SI values recorded by the experimental laboratories for each of the test chemicals are summarized in Tables 3(a) and 3(b), and the dose–response relationships for the SI values are indicated in Fig. 1(a) and (b).

The results of the first study are shown in Table 3(a) and Fig. 1(a). For chemicals A (2,4-dinitrochlorobenzene), B (hexyl cinnamic aldehyde), F (isoeugenol), and K (abietic acid), dose–response relationships of the SI values were clearly evident in each laboratory, and the SI values for all the high-dose groups were greater than 3. The dose–response relationships for chemicals H (dimethyl isophthalate), I (isopropanol), and L (methyl salicylate) were unclear, and the laboratories that assessed these chemicals reported negative findings. The SI values obtained for chemical C (3-aminophenol) in all 3 laboratories were lower than 3, and the values obtained in laboratories 1 and 3 were approximately 3 for the high-dose group. Further, dose–response relationships of the SI values were observed for chemicals D (glutaraldehyde) and G (formaldehyde), whose SI values were also approximately 3 for the high-dose groups. The SI values were greater than 3 for the high-dose groups in laboratories 1 and 2 but not in laboratory 5. The SI values for chemicals E (cobalt chloride) and J (nickel sulfate) were inconsistent across laboratories; further, an inconsistency was observed in the ATP content values in the vehicle control group for these chemicals. In the case of chemical E, the dose–response relationship of the weighted average of the SI values yielded a v-shaped curve; therefore, it may be considered that the observed dose–response relationships based on the weighted average values for chemical E were inappropriate.

Table 3(b) and Fig. 1(b) describe the results of the second study. For chemicals B (hexyl cinnamic aldehyde) and N (potassium dichromate),

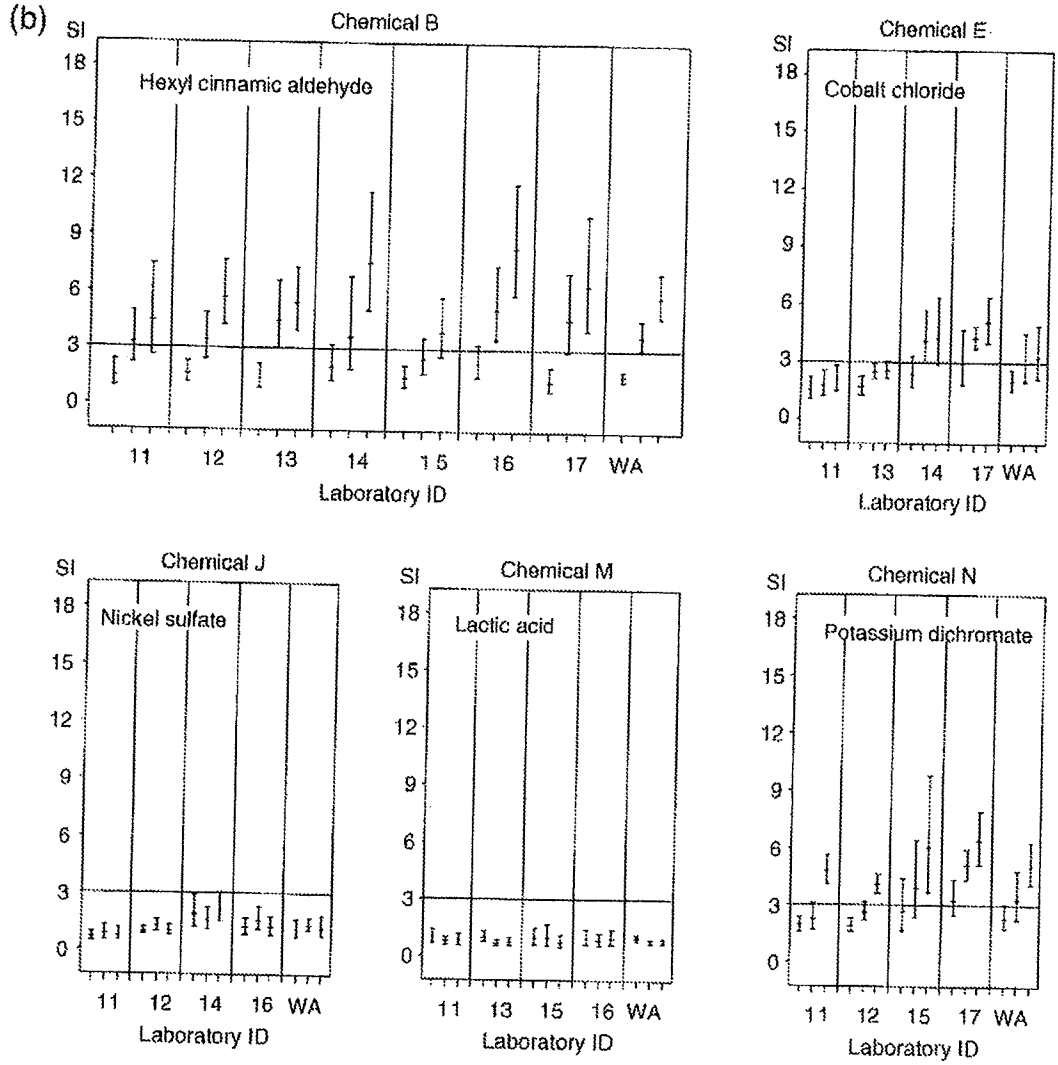

Fig. 1 (continued).

the dose–response relationships of the SI values were evident in each laboratory, and all the SI values of the high-dose groups were greater than 3. The SI values for chemicals J (nickel sulfate) and M (lactic acid) were lower than 3, and these chemicals tested negative in all the laboratories. The SI value for chemical E (cobalt chloride), which was inconsistent in the first study, was also inconsistent between different laboratories in the study. However, as opposed to the results of the first study, the dose–response relationships and ATP contents were considerably similar between laboratories.

3.4. ATP content and LNW

Fig. 2(a) and (b) shows the scatter plots of ATP content according to LNW for all the chemicals. Since the ATP content decreases with time, it is important for the scatter plot to demonstrate a linear relationship between the ATP content and LNW. This linear relationship can be used as a rough indicator of whether the experiments conformed to the protocol for measuring the ATP content. Since all the scatter plots demonstrated linearity, it can be concluded that all the experiments adhered to the protocol.

3.5. Assay sensitivity

We defined assay sensitivity as the ability to accurately detect the positive control chemical. Since a positive control was included in each experiment, we investigated whether the SI value assigned to the positive control group was greater than 3 in the experiments. Fig. 3(a) and (b) shows the SI values obtained for all the positive control groups with 95% confidence intervals. All the experiments in these studies were assay sensitive because all the SI values were greater than 3.

3.6. Intralaboratory variability

Although limited, the results obtained for the positive control groups allowed us to evaluate the intralaboratory variability of the assay. Fig. 3(a) and (b) also shows the variability of the SI values obtained for the positive control groups in each laboratory in both the studies. No large intralaboratory variation was observed in any of the laboratories.

3.7. Interlaboratory variability

The data shown in Fig. 1(a) and (b) were used to measure the interlaboratory variability in the SI values for all the chemical doses. Tables 4(a) and 4(b) show the weighted average of the SI values with 95% confidence intervals and a summary index of the interlaboratory variability, i.e., τ^2.

In the first study, all the doses of chemicals E (cobalt chloride) and J (nickel sulfate) and the intermediate dose of chemical D (glutaraldehyde) exhibited relatively large interlaboratory variations. On the

other hand, in the second study, no large interlaboratory variation was observed in any of the laboratories.

Tables 5(a) and 5(b) show the results of the judgments based on the cut-off value of 3 for the SI values obtained for all the chemicals in all the laboratories. In the first study, 4 chemicals, namely, D (glutaraldehyde), E (cobalt chloride), G (formaldehyde), and J (nickel sulfate), showed inconsistent results among the laboratories. For chemicals D (glutaraldehyde) and G (formaldehyde), the SI values for the high doses were approximately 3 among all 3 laboratories; thus, the variation was small. On the other hand, the values for chemicals E (cobalt chloride) and J (nickel sulfate) were inconsistent among the laboratories (Fig. 1(a)). In the second study, consistent results were observed for the 4 chemicals. Although an inconsistency was observed for chemical E (cobalt chloride), the dose-

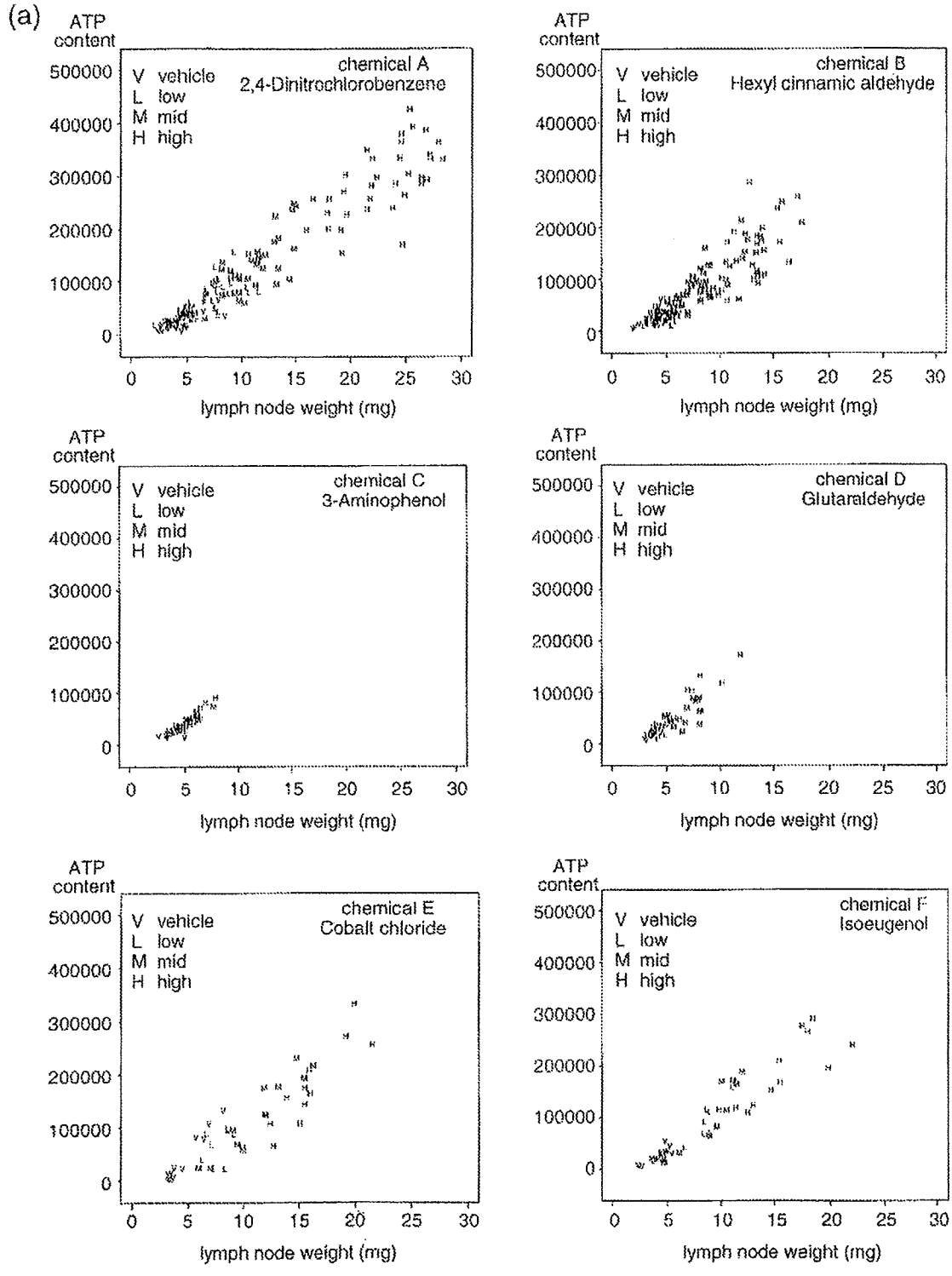

Fig. 2. (a). Scatter plots indicating the ATP content with the LNW (mg) recorded for the vehicle (V), low-dose (L), middle-dose (M), and high-dose groups (H) for each chemical in the first study. (b). Scatter plots indicating the ATP content with the LNW (mg) recorded for the vehicle (V), low-dose (L), middle-dose (M), and high-dose groups (H) for each chemical in the second study.

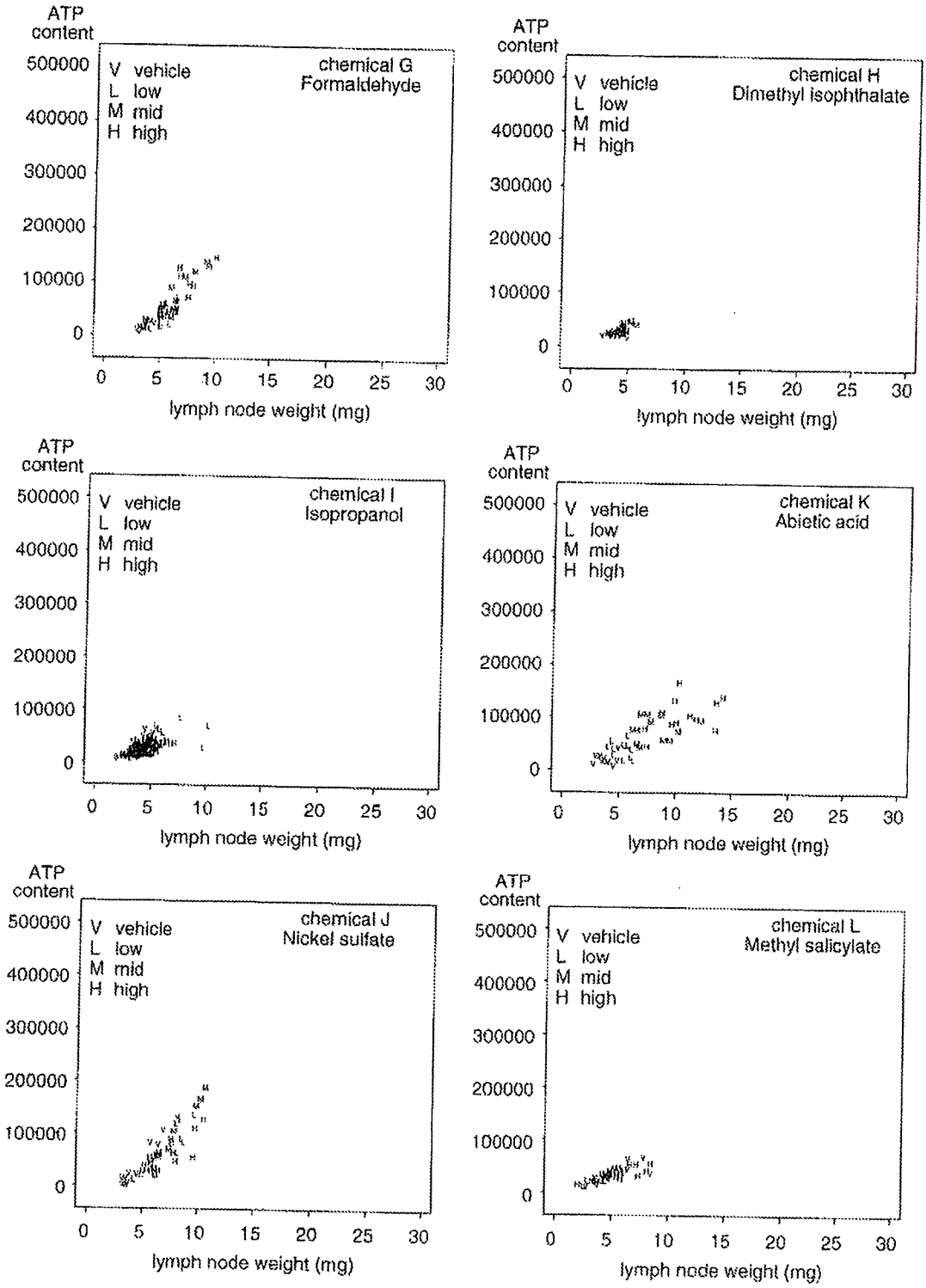

Fig. 2 (continued).

response relationships of the chemical were very similar between laboratories and the variation in the SI value for each dose was small, as mentioned above.

3.8. EC3 and measures of relevance

To avoid the problem of multiple counts of the same chemicals from different laboratories, the calculations of EC3 and sensitivity, specificity, accuracy, positive predictivity, and negative predictivity of LLNA-DA were based on the weighted averages of the SI values.

Tables 6(a) and 6(b) show the EC3 results and its classification for LLNA-DA based on the weighted averages for both the studies and the reported EC3 and its classification based on the reported values for LLNA.

The sensitivity, specificity, accuracy, positive predictivity, and negative predictivity of LLNA-DA with regard to the chemicals in the first study, as against those of GPMT/BT and LLNA are shown in Table 7.

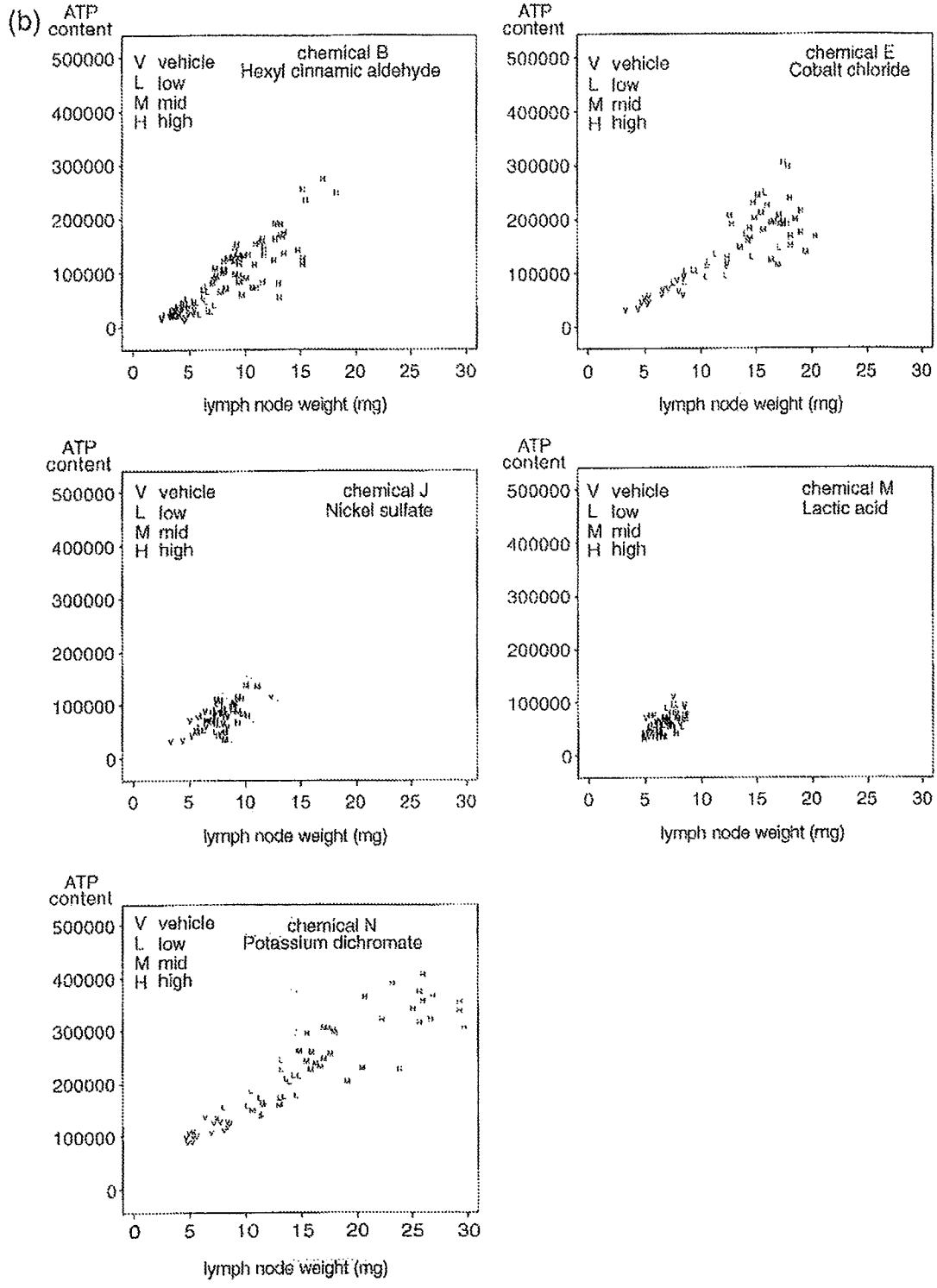

Fig. 2 (*continued*).

To enable comparison of the measurements of LLNA against those of GPMT/BT when the same chemicals were selected, these values calculated only on the basis of the referenced data are shown in Table 7.

The sensitivity, specificity, accuracy, positive predictivity, and negative predictivity values of LLNA-DA against those of GPMT/BT were similar to those of LLNA against those of GPMT/BT. Chemical C (3-aminophenol) was negative for LLNA-DA and positive for LLNA, and chemical J (nickel sulfate) was positive for LLNA-DA and negative for LLNA.

4. Discussion

Researchers have provided considerable evidence for the reliability of LLNA; however, limited evidence is available for the reliability of LLNA-DA. Since the methods involved in LLNA-DA and LLNA are essentially identical, the results of our study provide adequate evidence in support of LLNA-DA as an alternative assay method to LLNA.

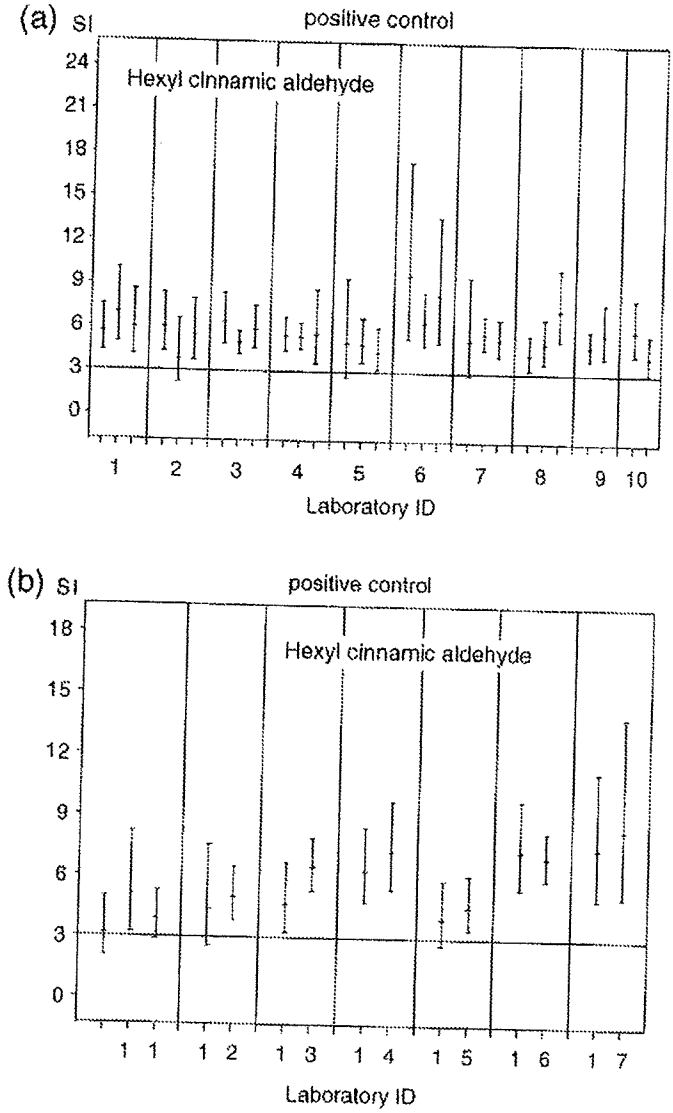

Fig. 3. (a). SI values with 95% confidence intervals obtained for the positive control (25% hexyl cinnamic aldehyde) groups in all the laboratories in the first study. (b). SI values with 95% confidence intervals obtained for the positive control (25% hexyl cinnamic aldehyde) groups in all the laboratories in the second study.

Although several interlaboratory studies on LLNA have been reported, they did not necessarily employ the same protocol; additionally, these studies were conducted by approximately 5 experimental laboratories (Basketter et al., 1991; Kimber et al., 1991, 1998, 1995; Loveless et al., 1996; Scholes et al., 1992). In contrast, one of the distinguishing features of the series of the present 2 studies is that 17 independent experimental laboratories used the same protocol to test chemicals. The fact that the interlaboratory variations were small for most of the chemicals is considered as a significant finding of this study. In particular, chemical B (hexyl cinnamic aldehyde) was tested by all the 17 laboratories; it was observed that the SI value of the interlaboratory variation was small and that the dose–response relationship was considerably similar. These results indicate that LLNA-DA is a robust technique.

In the first study, 2 of the 12 chemicals—chemicals E (cobalt chloride) and J (nickel sulfate)—demonstrated large interlaboratory variations. We considered that this might be attributed to the use of DMSO as the exclusive vehicle for these 2 chemicals. The fact that these 2 chemicals were the only metallic salts could be another reason for the large variations observed. Therefore, in the second study, it was necessary to examine interlaboratory variations with regard to other metallic salts with DMSO as the vehicle. The results of the second study, which used 5 chemicals including these 2 metallic salts, demonstrated small interlaboratory variations for all the chemicals. The small variation observed for the metallic salts could be due to the following reasons. (1) Prior to the study, the developer advised the toxicologists to carefully apply the DMSO solution onto the ears since it is highly hydrophilic, and the presence of moisture in the ears could lead to considerable variation in the results. (2) During the technical-transfer seminar, the participating toxicologists were well trained in all aspects of the experiment, including the application of DMSO solution onto the ears of the mice. Thus, our present finding is that the large variation for the 2 metallic salts in the first study was caused by inappropriate DMSO application, which in comparison with AOO or ACE has unique physical properties in terms of the difficulty involved in its application to the dorsum of the ears. Therefore, this factor was considered when the metallic salts were assessed with LLNA-DA.

Furthermore, these 2 studies provided substantial historical data with regard to the ATP content for the vehicle control group that used AOO, ACE, or DMSO with LLNA-DA. These data could be referred to by new laboratories that are considering the use of this assay. As in the case of LLNA studies, data from these studies regarding DMSO appear to demonstrate the toxicity of the chemical (Wright et al., 2001). We observed a higher ATP content when DMSO was used as a solvent than when AOO or ACE was used. This tendency may cause the SI values to change depending on the vehicle used in the experiment because a high ATP content in the vehicle control group would lead to relatively low SI values.

These studies also present certain limitations. First, the results are representative of only 14 chemicals. Although it may be recommended that the assay be tested using several chemicals, the

Table 4(a)
The weighted average of the SI values and the variance component, τ^2, in the first study

Chemical	Low-dose group			Middle-dose group			High-dose group		
	SI	95%CI	τ^2	SI	95%CI	τ^2	SI	95% CI	τ^2
A: 2,4-Dinitrochlorobenzene	2.5	(2.0, 3.0)	0.03	3.9	(3.1, 4.8)	0.03	10.3	(8.4, 12.8)	0.04
B: Hexyl cinnamic aldehyde	1.4	(1.2, 1.6)	0.00	2.8	(2.5, 3.3)	0.01	5.1	(4.2, 6.2)	0.03
C: 3-Aminophenol	1.4	(1.2, 1.7)	0.00	2.0	(1.6, 2.4)	0.00	2.2	(1.7, 2.9)	0.02
D: Glutaraldehyde	1.0	(0.7, 1.5)	0.02	2.3	(1.2, 4.6)	0.13	3.6	(2.4, 5.2)	0.03
E: Cobalt chloride	6.1	(2.7, 13.9)	0.11	5.0	(1.9, 13.2)	0.29	7.4	(2.4, 23.3)	0.42
F: Isoeugenol	2.7	(2.2, 3.4)	0.00	3.4	(2.4, 4.7)	0.02	6.7	(5.5, 8.3)	0.00
G: Formaldehyde	1.8	(1.1, 3.1)	0.07	2.7	(2.1, 3.4)	0.00	3.4	(2.5, 4.7)	0.01
H: Dimethyl isophthalate	1.2	(1.0, 1.4)	0.00	1.1	(0.9, 1.3)	0.00	0.9	(0.7, 1.2)	0.02
I: Isopropanol	1.1	(0.8, 1.4)	0.04	0.9	(0.8, 1.0)	0.00	0.9	(0.8, 1.1)	0.03
J: Nickel sulfate	2.7	(1.1, 6.5)	0.24	3.1	(1.4, 6.9)	0.20	3.1	(0.8, 12.1)	0.62
K: Abietic acid	2.1	(1.8, 2.4)	0.00	3.7	(3.1, 4.3)	0.00	5.4	(3.5, 8.3)	0.04
L: Methyl salicylate	0.9	(0.6, 1.3)	0.03	1.1	(0.7, 1.6)	0.04	1.2	(0.8, 1.9)	0.04

The variance component τ^2 represents the interlaboratory variance for the log-transformed SI, which is obtained by decomposing the total variance into the between variance and within variance by performing meta-analysis with a random effect model. Since τ^2 indicates variance, its value is greater than 0, and a higher value indicates greater interlaboratory variation.

Table 4(b)
The weighted averages of the SI values and the variance component,(τ^2, in the second study

Chemical	Low-dose group			Middle-dose group			High-dose group		
	SI	95%CI	τ^2	SI	95%CI	τ^2	SI	95% CI	τ^2
B: Hexyl cinnamic aldehyde	1.7	(1.4, 2.0)	0.00	3.8	(3.1, 4.6)	0.01	5.9	(4.8, 7.2)	0.01
E: Cobalt chloride	2.0	(1.5, 2.6)	0.02	3.0	(2.0, 4.5)	0.07	3.2	(2.1, 4.9)	0.07
J: Nickel sulfate	1.1	(0.7, 1.6)	0.06	1.3	(1.0, 1.6)	0.01	1.2	(0.8, 1.8)	0.07
M: Lactic acid	1.0	(0.8, 1.1)	0.00	0.7	(0.6, 0.9)	0.00	0.8	(0.7, 0.9)	0.00
N: Potassium dichromate	2.3	(1.8, 3.0)	0.02	3.3	(2.2, 4.8)	0.06	5.1	(4.1, 6.3)	0.02

The variance component τ^2 represents the interlaboratory variance for the log-transformed SI, which is obtained by decomposing the total variance into the between variance and within variance by using meta-analysis with a random effect model. Since τ^2 indicates variance, it takes on a value greater than 0, and a larger value indicates greater interlaboratory variation.

chemicals used in the present studies were selected from a wide range of chemicals, and their skin sensitization potentials were determined by the application of the LLNA method.

Further, the precision of the measurements of relevance was low because only 12 chemicals were tested by this assay method; therefore, even a difference in only a single chemical would affect the sensitivity. Since the study demonstrated the strong reliability of the assay, further assessments using other known chemicals should be conducted in other studies. Idehara et al. (in press) report the results of the intralaboratory study.

Another limitation is with regard to the quality of the data. It was extremely difficult to ensure complete compliance with good laboratory practice (GLP) in these studies. However, although the experiments involved in the studies were not conducted in complete accordance with GLP, the format file for data recording of individual experiments was devised at the planning stage of the study, and the data files collected for all the experiments complied with this format. Furthermore, since all the data used for the analyses were based on the database, if required, we can provide the database regarding the ATP content values obtained for the individual animals with the standard protocol that was used here.

Unlike LLNA, LLNA-DA measures the ATP content. It is an extremely simple method for measuring the ATP content during an experiment, and it yields quick results. However, since the ATP content of the LNCs decreases with time, while performing LLNA-DA, it is necessary to comply with the time of operation from lymph node excision to the determination of ATP content. Measuring the LNW as an internal control is recommended. The plot of ATP content against LNW, as in Fig. 2, might aid in roughly checking the compliance.

In conclusion, these 2 studies provide valuable evidence for the reliability of LLNA-DA.

Table 5(a)
Judgment based on SI values greater than 3 for LLNA and the referenced values for LLNA and GPMT/BT in the first study

Chemical	LLNA	GPMT/BT	Laboratory									
			1	2	3	4	5	6	7	8	9	10
A: 2,4-Dinitrochlorobenzene	+	+	+	+	+	+	+	+	+	+	+	+
B: Hexyl cinnamic aldehyde	+	+	+	+	+	+	+	+	+	+	+	+
C: 3-Aminophenol	+	+nonstd	−		−					−		
D: Glutaraldehyde	+		+	+			−					
E: Cobalt chloride	+	+				−		+		+		
F: Isoeugenol	+	+				+	+				+	
G: Formaldehyde	+	+	+	+			−					
H: Dimethyl isophthalate	−	−	−		−				−			
I: Isopropanol	−	−	−	−	−	−	−	−	−	−	−	−
J: Nickel sulfate	−	+				−		+		+		
K: Abietic acid	+	+		+				+	+			
L: Methyl salicylate	−	−			−				−			−

"nonstd" indicates a nonstandard animal.

Table 5(b)
Judgment based on SI values greater than 3 for LLNA and the referenced values for LLNA and GPMT/BT in the second study

Chemical	LLNA	GPMT/BT	Laboratory						
			11	12	13	14	15	16	17
B: Hexyl cinnamic aldehyde	+	+	+	+	+	+	+	+	+
E: Cobalt chloride	+	+	−		−	+			+
J: Nickel sulfate	−	+	−	−		−		−	
M: Lactic acid	−	−	−		−		−	−	
N: Potassium dichromate	+	+	+	+			+		+

Table 6(a)
EC3 and chemical classification in the first study

Chemical	LLNA-DA		LLNA	
	EC3	Classification	EC3	Classification
A: 2,4-Dinitrochlorobenzene	0.06	Extreme	0.04	Extreme
B: Hexyl cinnamic aldehyde	11.1	Weak	8.4	Moderate
C: 3-Aminophenol	–	Negative	3.2	Moderate
D: Glutaraldehyde	0.3	Strong	0.1	Extreme
E: Cobalt chloride	–	(Positive)[a]	<0.5	Strong
F: Isoeugenol	1.9	Moderate	1.8	Moderate
G: Formaldehyde	3.0	Moderate	0.7	Strong
H: Dimethyl isophthalate	–	Negative	–	Negative
I: Isopropanol	–	Negative	–	Negative
J: Nickel sulfate	2.7	Moderate	–	Negative
K: Abiet ic acid	7.9	Moderate	14.7	Weak
L: Methyl salicylate	–	Negative	–	Negative

The EC3 for LLNA-DA is based on the weighted average. The SI values obtained for chemical E (cobalt chloride) with LLNA-DA was greater than 3 for all the doses; however, since the dose–response relationship yielded a v-shaped curve, the EC3 could not be determined.

[a] Although the weighted averages of the SI values were greater than 3 for all the doses, the EC3 and classification were determined because the dose–response relationship exhibited a v-shaped curve.

Table 6(b)
EC3 and chemical classification in the second study

Chemical	LLNA-DA		LLNA	
	EC3	Classification	EC3	Classification
B: Hexyl cinnamic aldehyde	8.1	Moderate	8.4	Moderate
E: Cobalt chloride	3.0	Moderate	<0.5	Strong
J: Nickel sulfate	–	Negative	–	Negative
M: Lactic acid	–	Negative	>25	Negative
N: Potassium dicromate	0.2	Strong	0.1	Strong

Table 7
Sensitivity, specificity, accuracy, positive predictivity, and negative predictivity in the study

	n	Sensitivity	Specificity	Accuracy	Positive predictivity	Negative predictivity
LLNA-DA vs. GPMT/BT	11	87.5% (7/8)	100% (3/3)	90.9% (10/11)	100% (7/7)	75.0% (3/4)
LLNA-DA vs. LLNA	12	87.5% (7/8)	75.0% (3/4)	83.3% (10/12)	88% (7/8)	75.0% (3/4)
LLNA vs. GPMT/BT	11	87.5% (7/8)	100% (3/3)	90.9% (10/11)	100% (7/7)	75.0% (3/4)

For LLNA-DA, the judgment was based on the weighted average of the SI values. For LLNA and GPMT/BT, judgments were based on the referenced data.

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Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings

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ABSTRACT

The arylhydrocarbon receptor (AhR) mediates sex steroid hormone-related actions in both normal physiology and in dioxin toxicity. In addition to regulation of direct target genes, the ligand-activated AhR associates with estrogen or androgen receptors (ERα or AR) to regulate transcription as a functional unit. Given that endogenous and exogenous AhR-ligands are structurally diverse, it is unclear whether cross-talk regulation of ERα/AR by the activated AhR is an intrinsic function of the AhR or the result of ligand-type-selective differences. To ensure uniform activity of the AhR irrespective of ligand-type-specific differences, we employed CA-AhR, which lacks the ligand-binding domain and has a constitutive activity. We found that CA-AhR, in the absence of a ligand, acted as a transcriptional co-regulator for the unliganded ERα/AR as well as for mutants of ERα/AR lacking a ligand-binding domain. CA-AhR was recruited to estrogen-/androgen-responsive promoters with endogenous ERα/AR. Moreover, CA-AhR had an E3 ubiquitin ligase activity and promoted proteasomal degradation of ERα/AR. Thus, these findings indicate that the cross-talk function of the AhR with sex hormone receptors is an intrinsic function of the AhR.

The arylhydrocarbon receptor (AhR) is a member of the basic helix–loop–helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors. The AhR mediates the toxic effects of dioxins such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) [1–3], in part by modulating estrogen and androgen signaling [4,5]. AhR-deficient mice exhibit both abnormal estrous cycles and defective ovarian follicle maturation in females [6], and developmental defects in the prostate of males [7]. This implies a sex hormone-related innate function of the AhR.

The transcriptional activity of the AhR is primarily regulated by ligand-dependent translocation to the nucleus [3]. Unliganded AhR is sequestered in the cytosol by interacting with the Hsp90-XAP2 (also called AIP or ARA9) chaperon complex through the PAS-B region [1–3]. Ligand binding to the PAS-B region of the AhR induces dissociation of the Hsp90-XAP2 and subsequent translocation of the AhR to the nucleus[1–3]. The AhR then dimerizes with Arnt, recognizes the xenobiotic responsive element (XRE), and recruits co-activators [3]. The AhR induces expression of direct target genes such as the drug metabolizing enzymes CYP1A1 and CYP1A2 [1–3]. Disruption of inhibitory PAS-B function by ligand binding is therefore expected to be sufficient to induce transcriptional activity of the AhR [8]. In fact, a mutant AhR that lacks a PAS-B region (CA-AhR) is constitutively active and exhibits transcriptional activity irrespective of lack of ligand-binding capacity [9].

AhR exhibits other regulatory functions by modulating the function of other transcription factors, including Rb/E2F [10], NF-κB [11], and the estrogen (ERα and ERβ) [12–16] and androgen (AR) [16] receptors. These cross-talk pathways are important components that mediate the functions of endogenous and exogenous AhR-ligands. As for the estrogen-related adverse effects of dioxins, ligand-activated AhR/Arnt associates with ERα and ERβ through the N-terminal A/B region within the ERs [12–16]. By means of this association, the liganded AhR potentiates the transactivation function of 17β-estradiol (E_2)-unbound ERα and represses E_2-bound ERα-mediated transcription upon the estrogen-responsive element (ERE) [12]. Reciprocally, E_2-bound ERα associates with XRE-bound AhR to either potentiate [13] or repress [14] AhR-mediated transcription. Reflecting this functional cross-talk, Arnt also acts as a co-regulator for ERα [17]. In addition, it has been recently shown that the liganded AhR promotes ubiquitination and proteasomal degradation of ERα and the AR by assembling a ubiquitin ligase complex, $CUL4B^{AhR}$ [16]. Thus, complexes of the AhR with ERs or

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AR appear to regulate transcription as functional units by multiple mechanisms.

Several diverse types of AhR-ligands have been reported to date, including endogenous [tryptamine, indole-3-acetic acid, and indirubin], exogeneous [TCDD, and benzo(a)pyrene], and synthetic [3-methylcholanthrene (3MC), and β-naphthoflavone (βNF)] ligands [18]. Since these compounds are structurally diverse, and the physiological ligand(s) for the AhR has not been defined, it is unclear whether modulation of ERα and AR function by the liganded AhR may be attributed to an intrinsic function of the AhR.

To ensure uniform activity of the AhR in our experiments, irrespective of ligand-type-specific differences, we employed CA-AhR which lacks binding capacities for the Hsp90-XAP2 as well as for ligands [8]. CA-AhR has constitutive transcriptional activity in the absence of ligands [8], and CA-AhR transgenic mice exhibit dioxin-exposure-related phenotypes in various tissue such as the liver and stomach [9]. These characteristics render this mutant particularly suitable for studying the intrinsic function of the AhR irrespective of ligand-type-specific differences. In this study, we demonstrated that CA-AhR acts as a transcriptional co-regulator for the unliganded ERα/AR, even in the absence of AhR-ligands. Moreover, CA-AhR promoted proteasomal degradation of both ERα and the AR. Therefore, these findings indicate that cross-talk with the sex hormone receptors is an intrinsic function of the AhR that is not subject to ligand-type-selective differences.

Materials and methods

Plasmids. Expression plasmids for the AhR, Arnt, constitutively active AhR, ERα, ERαΔD/E/F, AR, and ARΔE/F were previously described [12]. Luciferase reporter plasmids [estrogen-responsive element (ERE)-Luciferase, androgen-responsive element (ARE)-Luciferase, and xenobiotics-responsive element (XRE)-Luciferase] were described previously [12].

Cells, transfection, and luciferase assay. Human breast cancer-derived MCF-7 cells, human endometrial cancer-derived Ishikawa cells, human prostate cancer-derived LNCaP cells, and human renal cancer-derived 293T cells were cultured as previously described [16]. Cells were cultured in phenol-red-free DMEM containing 0.2% charcoal-stripped FBS and transfected with the receptor expression vectors and the luciferase reporter plasmids [16]. Cells were then treated with E_2 (10 nM), DHT (10 nM), 3-methylcholanthrene (3MC) (1 μM), β-naphthoflavone (β-NF) (1 μM), or MG132 (10 μM), for 24 h (Luciferase assays) or for 3 h (Western blotting).

For the Luciferase assays [19], cells at 40–50% confluence were transfected with the indicated plasmids [0.25 μg reporter plasmids, 0.025 μg ERα, 0.1 μg ERαΔD/E/F, AhR/Arnt (+, 0.05 μg; ++, 0.1 μg; +++, 0.2 μg), 0.1 μg AR, 0.1 μg ARΔE/F] using Lipofectamine reagent (Gibco–BRL). Luciferase activity was determined with the Luciferase assay system (Promega). As a reference plasmid to normalize transfection efficiency, 2.5 ng pRL-CMV plasmid (Promega) was co-transfected. All values represent averages ±SD of at least three independent experiments.

In vitro ubiquitination assay. The *in vitro* ubiquitination assay was performed as previously described, with several modifications [16]. The CA-AhR immunocomplex was purified using anti-FLAG antibody from MCF-7 cells transfected with FLAG-HA-CA-AhR together with HA-DDB1 and myc-TBL3. The immunocomplex was incubated with recombinant ubiquitin and reaction buffer [50 mM Tris–HCl (pH 7.4), 5 mM MgCl, 2 mM NaF, 2 mM ATP and ATP-regenerating system, 0.6 mM DTT, and 12 μg ubiquitin (Calbiochem), 60 ng E1(Calbiochem), 0.3 μg E2 mixture set (Calbiochem)]. The self-ubiquitination of CA-AhR was detected by Western blotting using an anti-HA antibody.

ChIP assays. ChIP assays were performed essentially as previously described, with several modifications [12]. MCF-7 or LNCaP cells were transfected with 5 μg FLAG-CA-AhR in a 100 mm dish. After 36 h, the cells were treated with a transcription inhibitor α-amanitin (2.5 μM) for 2 h, released by a washing twice with PBS for 10 min and medium change. After the medium change, the cells were cross-linked at the indicated time (min).

More detailed methods are supplied as Supplementary information.

Results and discussion

CA-AhR activates the transcriptional function of unliganded ERα and AR

First, we tested if CA-AhR was indeed constitutively active in a xenobiotic-responsive element (XRE)-driven reporter assay in endometrial tumor-derived Ishikawa cells. CA-AhR activated XRE-mediated transcription in the absence of AhR-ligand as efficiently as 3MC-bound wild-type AhR (Supplementary Fig. 1).

Under this experimental condition, the effects of CA-AhR on ERα- and AR-mediated transcription were examined with the reporter assays. A reporter plasmid containing either a consensus estrogen-responsive element (ERE) or a consensus androgen-responsive element (ARE) was co-transfected with different amounts of either wild-type AhR or CA-AhR into Ishikawa cells (for ERE assays) or kidney-derived 293T cells (for ARE assays). When the wild-type AhR was transfected, transfection-dosage-dependent activation of the ERE- or ARE-Luciferase activity in the presence of, but not in the absence of, AhR-ligands [3MC and βNF], was observed (Fig. 1A and B). In that reporter assay, we found that CA-AhR significantly activated the ERE- or ARE-Luciferase activity in a transfection dosage-dependent manner in the absence of AhR-ligands (Fig. 1A and B). The activation function of CA-AhR for ERα-/AR-mediated transcription was comparable with that of the ligand-bound wild-type AhR, as expression levels of these AhR derivatives were not significantly different (Fig. 1C). We obtained similar results on the observed CA-AhR modulation of ERα function in mammary tumor-derived MCF-7 cells (data not shown).

CA-AhR activates ERα and AR mutants which lack C-terminal ligand-binding domains

To further demonstrate direct modulation of ERα-/AR-mediated transcription by the AhR, we employed ERα and AR mutants which lack C-terminal ligand-binding domains (LBDs; also known as E/F regions) [12]. Nuclear receptors exhibit two activation functions (AFs), AF-1 and AF-2 [20]. The AF-1 transactivation function in the A/B domain is ligand-independent, while the AF-2 function in the LBD is induced in a ligand-dependent manner through conformational change [20]. The LBD-deficient ERα and AR derivatives [ERαΔD/E/F and ARΔE/F] have DNA-binding activity but retain only AF-1 function [12].

As shown in lane 2 of Fig. 2A and B, transfected ERαΔD/E/F and AR ΔE/F mutants exhibited AF-1 activity, which was unaffected by the presence of E_2 or DHT, respectively (Fig. 2A and B, lane 12). Nonetheless, CA-AhR activated ERαΔD/E/F- and AR ΔE/F-mediated transcription in a transfection dosage-dependent manner in the absence of ERαAR ligands (lanes 9–11). The wild-type AhR, however, activated ERαΔD/E/F- and AR ΔE/F-mediated transcription only in the presence of 3MC (lanes 6–8). Importantly, since neither CA-AhR nor the LBD mutant of ERαAR has a functional ligand-binding domain, it is highly unlikely that the modulation of ERαΔD/E/F and AR ΔE/F by CA-AhR can be attributed to overlapping ligand-responsibility.

CA-AhR is recruited to estrogen- and androgen-responsive promoters in the absence of ligands

To explore the function of CA-AhR in the endogenous chromatin context, we tested whether CA-AhR was functional in endogenous estrogen-/androgen-responsive promoters by a chromatin immunoprecipitation (ChIP) assay. For this purpose, we used MCF-7 cells and LNCaP cells. Endogenous ERα and AR functionally bind to estrogen-responsive *c-fos* promoters in MCF-7 cells and androgen-responsive *PSA* promoters in LNCaP cells, respectively [12,21]. The wild-type AhR, upon ligand treatment, is recruited to the XRE-containing promoters at 30–60 min, and dissociates from the promoters after 60 min. The ligand-activated AhR is recruited to and dissociates from estrogen-responsive promoters together with ERα on a similar time course [12]. In order to synchronize the transcriptional cycle of CA-AhR in the absence of ligands, we

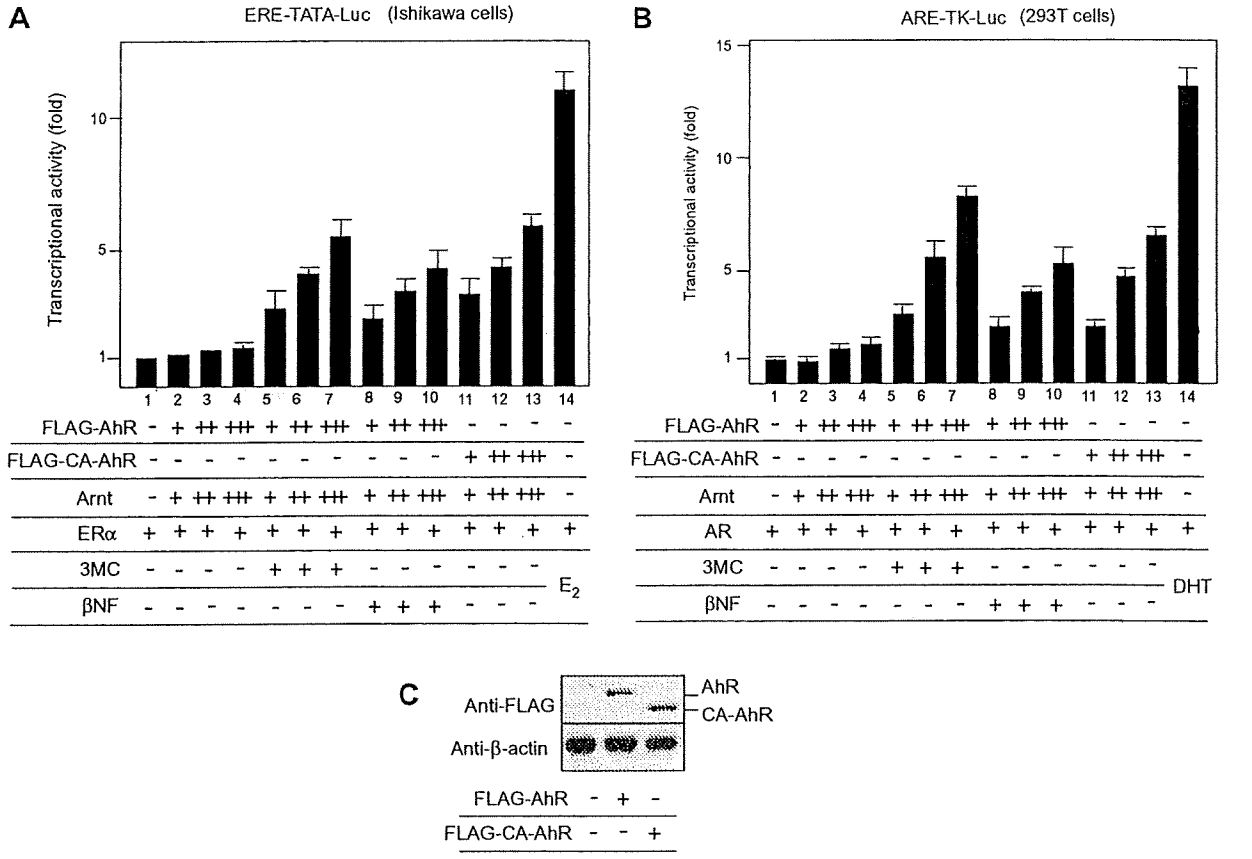

Fig. 1. The constitutively active AhR activates transcription through the unliganded ERα and AR in the absence of AhR-ligands. Ishikawa cells (A) or 293T cells (B) were transfected with the reporter plasmid bearing ERE (A) or ARE (B) together with the indicated expression plasmids (AhR/Arnt; +, 0.05 μg; ++, 0.1 μg; +++, 0.2 μg), in the presence or absence of the indicated ligands (3MC, 1 μM; βNF, 1 μM), and a Luciferase assay performed. Data are means ± SD of three independent experiments. (C) 293T cells were transfected with the indicated expression vectors (0.5 μg for FLAG-AhR and FLAG-CA-AhR), and the lysates subjected to Western blotting.

used the α-amanitin-release method, which is widely used in similar time-course ChIP experiments [22]. The MCF-7 cells and LNCaP cells transfected with CA-AhR were treated with α-amanitin, a transcription inhibitor, for two hours, and were then released by washing and a medium change.

Upon release from α-amanitin, CA-AhR was recruited to the *c-fos* promoter at 30–60 min in MCF-7 cells. Interestingly, endogenous ERα was co-recruited with CA-AhR to the *c-fos* promoter following a similar time-course in CA-AhR-transfected cells. No significant recruitment of ERα was observed 60 min after α-amanitin release in non-transfected cells (Fig. 3A). Similarly, CA-AhR was recruited to the *PSA* promoter at 60 min in LNCaP cells, and transfection of CA-AhR induced co-recruitment of endogenous AR on the *PSA* promoter on a similar time-course as CA-AhR (Fig. 3B). These results suggest that CA-AhR associates with endogenous ERα or AR on estrogen- or androgen-responsive promoters, respectively, as a transcriptional co-regulator.

CA-AhR has ubiquitin ligase activity and promotes proteasomal degradation of ERα in the absence of ligands

Finally, we tested if CA-AhR acted as an E3 ubiquitin ligase in the absence of a ligand. The ligand-activated AhR assembles a $CUL4B^{AhR}$ complex consisting of CUL4B/DDB1/Rbx1/TBL3/AhR/Arnt [16]. It recognizes ERα and the AR and promotes their ubiquitin–proteasome-mediated degradation [16]. Therefore, in this study the ubiquitin ligase activity of CA-AhR was verified. The CA-AhR immunocomplex, which included the components of $CUL4B^{AhR}$, was prepared, and the *in vitro* ubiquitination assay performed. The CA-AhR complex has an E3 ubiquitin ligase activity that is E1/E2-enzyme-dependent *in vitro*, as revealed by its self-ubiquitination activity (Fig. 4A).

The effects of CA-AhR on the degradation of ERα and the AR were then examined. ERα was co-transfected with either the wild-type AhR or CA-AhR into MCF-7 cells. After incubation with ligands and/or a proteasomal inhibitor MG132 for 6 h, cell lysates were prepared and the protein levels of ERα measured with Western blotting. To avoid detection of endogenous ERα protein in the non-transfected cells, we transfected FLAG-tagged ERα and detected ERα with a FLAG antibody. As shown in Fig. 4B, CA-AhR promoted degradation of ERα in the absence of the AhR-ligand in a transfection dosage-dependent manner (Fig. 4B, lanes 4 and 5). In contrast, wild-type AhR promoted degradation of ERα only in the presence of the AhR-ligand (lanes 2 and 3). A proteasomal inhibitor MG132 abolished the promotion of degradation, confirming a ubiquitin–proteasome pathway mediated mechanism (lane 6). Proteasomal degradation of the wild-type AhR itself was promoted by the AhR-ligand (lanes 2 and 3), consistent with previous reports [1–3].

Consistently, when the AR was co-transfected with either the wild-type AhR or CA-AhR into 293T cells, we found that CA-AhR promoted proteasomal degradation of the AR in the absence of AhR-ligand (Fig. 4C). The wild-type AhR promoted degradation of the AR only in the presence of a ligand (data not shown). These re-

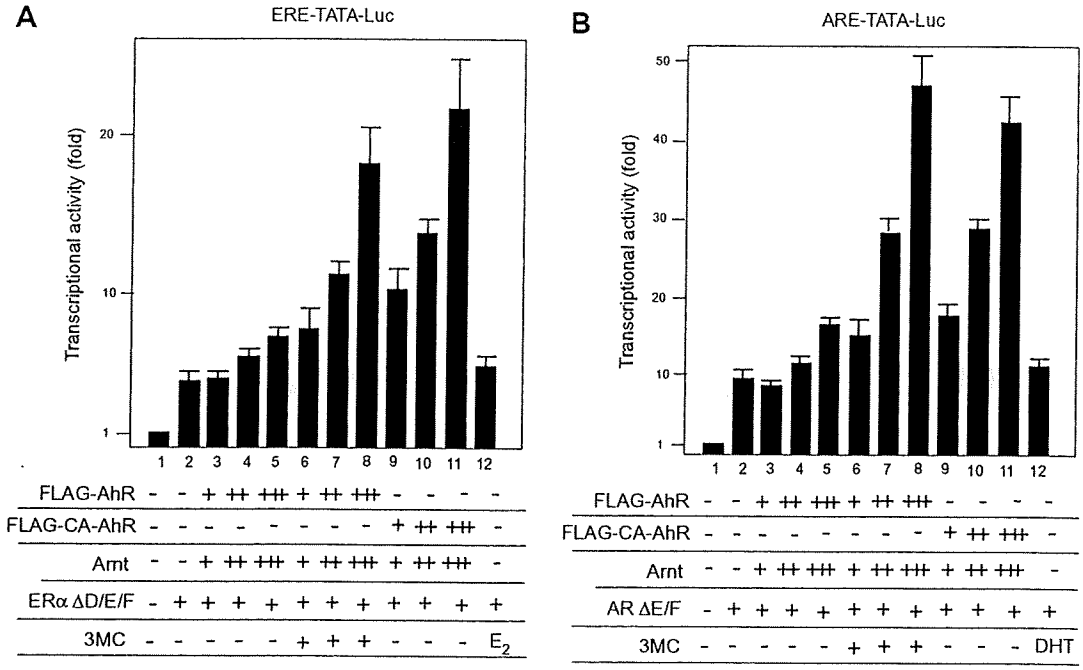

Fig. 2. CA-AhR activates mutants of ERα and AR which lack ligand-binding domains. Ishikawa cells (A) or 293T cells (B) were transfected with the indicated plasmids, and the Luciferase assay was performed as in Fig. 1. Data are means ± SD of three independent experiments. The data show that CA-AhR modulates the transcriptional activity of ERαΔD/E/F and AR ΔE/F in the absence of ligands.

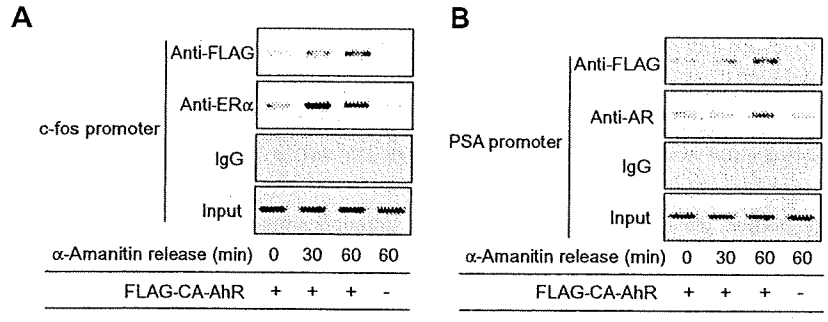

Fig. 3. Promoter occupancy of CA-AhR together with ERα or the AR in the absence of AhR-ligands. MCF-7 cells (A) or LNCaP cells (B), in which endogenous ERα (A) or the AR (B) was functional, were transfected with FLAG-tagged AhR or CA-AhR as indicated. The cells were treated with 10 μM α-amanitin for 2 h, and then subjected to the ChIP assay at the indicated time (min) after release from α-amanitin by a medium change. Samples were immunoprecipitated with the indicated antibodies, and promoter DNAs were amplified by PCR as indicated.

sults suggest that the activated AhR directly modulates stability of ERα and AR proteins by its ubiquitin ligase activity, irrespective of the ligand.

AhR as a transcriptional co-regulator for ERα/AR

Accumulating evidence suggests some of the actions of the AhR are mediated through cross-talk pathways with other transcription factors, including Rb [10], NF-κB [11,23], and nuclear receptors [5,12–16]. We and other groups have previously reported that the ligand-activated AhR directly associates with ERα and the AR to regulate transcription [12–16]. Moreover, AhR-dependent degradation of ERα has been independently reported [15,16]. To provide additional evidence for these AhR-ERα/AR cross-talk pathways, we have shown that CA-AhR, which lacks the ligand-binding domain and has a constitutive activity [8,9], modulates the functions of ERα and the AR in the absence of AhR-ligands. This suggests that activation of the AhR, either by ligand binding or by deletion of an inhibitory domain, directly induces a regulatory AhR-ERα/AR cross-talk pathway. Moreover, we have shown that the ubiquitin ligase activity of CA-AhR is also intact in the absence of a ligand. These results suggest that modulation of ERα and the AR is an intrinsic function of the AhR.

Importantly, we have shown that the mutants of ERα and AR which lack the C-terminal ligand-binding domains are also functionally regulated by CA-AhR. This directly excludes the possibility of cross-binding of AhR-ligands to ERα and the AR. Consistent with this, it was recently demonstrated that the AhR-ligand 3MC does not directly bind to ERα [24]. Together, these results indicate that the active form of the AhR, but not the ligand itself, is required for AhR-ERα/AR cross-talk. Thus, the present data support the existence of a 'direct cross-talk' pathway in which the AhR directly associates with ERα/AR and regulates their function as a transcriptional co-regulator. In the nuclear receptor superfamily of tran-

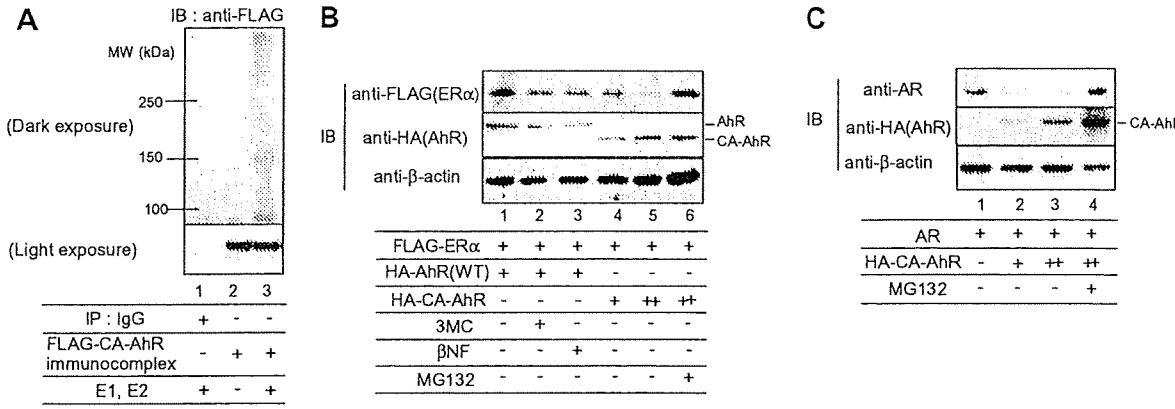

Fig. 4. CA-AhR promotes proteasomal degradation of ERα and the AR. (A) A FLAG-CA-AhR immunocomplex and IgG immunocomplex, prepared from MCF-7 cells, were subjected to the *in vitro* ubiquitination assay. The self-ubiquitination of CA-AhR was detected by Western blotting as indicated. (B,C) MCF-7 cells (B) or 293T cells (C) were transfected with the indicated plasmids [0.25 μg FLAG-ERα, 0.25 μg AR, 0.5 μg HA-AhR, HA-CA-AhR (+, 0.5 μg; ++, 1.0 μg) in 6-well dish]. Twenty-four hours after transfection, the cells were incubated with the indicated ligands or vehicle, then lysed and subjected to Western blotting with the indicated antibodies.

scription factors, ligand-type-selective differences in receptor conformational change affect the interaction with specific co-regulators [21,25]. Given that AhR-ligands are structurally diverse, ligand-specific differences in the modulation of AhR conformation may result in differential interaction with ERα/AR, CUL4B, and co-activators such as p300. This may lead to differential regulation of cross-talk pathways.

In the cross-talk pathways, AhR, like other co-regulators [26], both positively and negatively regulates other transcription factors. For instance, AhR associates with Rb/E2F1 and cooperatively regulates transcription, both positively [27,28] and negatively [10]. Similarly, AhR and NF-κB pathways converge in either a cooperative [11] or inhibitory [23] manner, depending on the cellular conditions. Our presented data indicate that the AhR assembles both a transcriptional co-regulator complex and a ubiquitin ligase complex. These complexes may explain the bi-phasic functions of the AhR-ligand in the regulation of other transcription factors, and are likely related to the physiological function of the AhR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.054.

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Review

Safety assessment of biopharmaceuticals: Japanese perspective on ICH S6 guideline maintenance

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ABSTRACT — Safety assessment of biopharmaceuticals in preclinical studies is guided by the ICH S6 guideline issued in 1997. Along with enormous experiences and knowledge on safety assessment of some classes of biopharmaceuticals over the last decade, the necessity and feasibility of updating the guideline has been discussed. According to a recommendation by safety experts at the ICH meeting in Chicago in 2006, regional discussions of ICH S6 were held in the USA, EU and Japan. The meeting to clarify the values, challenges and recommendations for ICH S6 from Japanese perspective was held as a part of the first Drug Evaluation Forum in Tokyo on August 10, 2007. Of utmost importance, the "case-by-case" approach must be preserved as the basic principle of the ICH S6 guideline. It is our opinion that oligonucleotides, siRNA, aptamers and related molecules should be excluded from ICH S6 and may be more appropriate for separate guidance. However, based on experiences and accumulated knowledge, there are a number of issues that can be updated including new types of biopharmaceuticals such as bioconjugates, use of homologous proteins and transgenic animals, reproductive/developmental toxicity studies in non-human primates, *in vitro* cardiac ion channel assay and alternative approaches for carcinogenicity assessment. Preliminary recommendations for some of these topics were outlined at the meeting. The overall Japanese recommendation is that the ICH S6 guideline should be updated to address these topics.

Key words: ICH S6 guideline, Biopharmaceutical, Safety assessment, Preclinical

INTRODUCTION

Biotechnology-derived pharmaceuticals (biopharmaceuticals) appeared for the first time in the 1980s, and the numbers of biopharmaceuticals in the market and in development have increased dramatically over the last two decades. A number of concerns/questions were raised in the early 1990s about the scientific justifications for the safety assessment of biopharmaceuticals in preclinical studies, since preclinical safety guidelines for small molecular new chemical entities (NCEs) are usually not appropriate for biopharmaceuticals. To answer some of those questions, the ICH S6 guideline was issued in 1997. The ICH S6 guideline stresses the principle that preclin-

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ical safety evaluation of biopharmaceuticals should be addressed on a "case-by-case" basis. The "case-by-case" approach means that the design and evaluation of safety studies is justified based on an appropriately understanding: (1) of the pharmacology across species, (2) that differences between biopharmaceuticals and NCEs require different endpoints and studies, and (3) that the class of biopharmaceutical influences the endpoints and studies. These principles are still valid and must continue to be preserved. However, enormous experience and knowledge on safety assessment of some classes of biopharmaceuticals has been accumulated while novel types of biopharmaceuticals continue to be developed. Furthermore, to help clarify the regional interpretations of ICH S6, local documents on the safety assessment of biopharmaceuticals have been written in the USA (FDA, 1997; FDA, 2000; Hastings, 2007), EU (CPMP/372/01, 2001; CPMP/SWP/2600/01, 2002; EMEA/CHMP/SWP/294648, 2007) and Japan (Pharmaceutical Non-clinical Investigation Group, 2002; Nakazawa *et al.*, 2004). It was agreed at the ICH Chicago meeting in 2006 that regional meetings in the EU, USA and Japan would be convened to address the potential need for updating the ICH S6 guideline. Future discussions were to be guided by the following key questions: 1) What can be learned from case studies and experience? 2) What is the predictive value of preclinical studies?; and 3) Where does the ICH S6 guideline "work" and/or "not work"? In addressing these questions, topics considered to be important were: new types of biopharmaceutiocals, such as bioconjugates and oligonucleotide medicines, initial dose for first in human study (FIH) selected from preclinical data, non-human primate developmental toxicity studies, *in vitro* cardiac testing, genotoxicity tests, carcinogenicity studies and the use of transgenic models and homologous products. The Japanese regional meeting was held at the first Drug Evaluation Forum in Tokyo on August 10, 2007. Experts from industry, regulatory bodies and academia participated in the meeting. This paper summarizes the Japanese perspective on values, challenges and recommendations for ICH S6 guidelines that emerged from the meeting.

VALUES, CHALLENGES AND RECOMMENDATIONS FOR ICH S6 GUIDELINE

General principle

1. Scope

The ICH S6 guideline was developed for pharmaceuticals derived from biotechnology, i.e. medical products of proteins/peptides and their analogues. It can also be applied to chemically synthesized peptides, most of which have properties similar to biopharmaceuticals as well as to bioconjugates (a protein combined with chemical molecule or a part or full molecule of other protein), although some special considerations are needed, as discussed in the sections of genotoxicity testing, human *ether-a-go-go* related gene (hERG) assay and carcinogenicity studies. In the event that there is a safety concern about a chemical fragment derived from a bioconjugate through degradation and/or metabolism, the concern should be addressed as a NCE. Such considerations for bioconjugates would be shared for protein/peptide analogs with non-natural amino acids. On the other hand, oligonucleotide medicines including antisense, RNAi and aptamers have very different physicochemical and biological properties from biopharmaceuticals, and therefore may need a new guideline for preclinical safety assessment.

2. Basic principle

The most important concept established by the ICH S6 guideline is the "case-by-case" approach. The underlying principle is that an appropriate safety test should be used for each biopharmaceutical considering the available information and the unique nature of each entity. Thus, it allows flexibility in designing the best safety assessment possible and discourages uniformed application of a standard list of studies designed for NCEs. The overwhelming consensus of the meeting was that the "case-by-case" concept must be preserved.

3. Species selection

It is very important to select relevant species for the safety assessment of a biopharmaceutical based on its pharmacological and/or biological activities. However, no relevant animal species are available in some cases. No clear advice is written in the ICH S6 guideline on when and how to use transgenic animals or homologous proteins, although the guideline recommends that these alternatives may assist in the safety assessment of biopharmaceuticals.

The use of homologous proteins to address species difference is more common than transgenic animals. However, it is important to consider that it takes months to years to make and characterize a homologue, and thus the sponsor needs to make a decision as early as possible whether or not a homologue is needed for safety assessment. As described in the ICH S6 guideline, the production process, range of impurities/contaminants, pharmacokinetics, and exact pharmacological mechanism(s) may differ between the homologous form and the product intended for clinical use. The comparability of the homologue with

the clinical candidate is critical for the interpretation of the toxicity results obtained with the homologue. Therefore, the sponsor should pay particular attentions to characterizing the pharmacology and pharmacokinetics of the homologue. For monoclonal antibodies, literature information, *in vitro* binding, function assays, tissue cross-reactivity and Fc activity are useful for the characterization.

Another important consideration when interpreting results using a homologue is the margin of safety. Even if negative findings are obtained with a homologue, the sponsor should still be cautious in the risk assessment of the clinical candidate. Conversely, if a homologue produces more severe toxicity in a rodent study compared to data using the clinical candidate in a monkey toxicity study, it is not a foregone conclusion that the results from rodent homologue studies take precedence over those with the clinical candidate. Additional factors need to be considered including that the homologue may have different pharmacokinetics and/or pharmacodynamics from the clinical candidate. Furthermore, the physiology of the target organ in a rodent can differ significantly from human. Finally, physiological similarity between the monkey and human may make the interpretation of the nonhuman primate studies more relevant to risk assessment of man. Thus, a sponsor should interpret the results from studies using a homologue using case-by-case considerations of all available scientific information, including comparability data between a homologue and clinical candidate, physiology across species and literature data with similar products. If a relevant animal species is available for the clinical candidate, a rodent study with a homologue usually is not needed.

4. Dose selection

The ICH S6 guideline recommends the dose selection for toxicity studies should take pharmacokinetics, pharmacodynamics and the expected clinical dose into consideration. The need for observable toxicity at the highest dose remains controversial for biopharmaceuticals. In some cases, only exaggerated pharmacological effects may be observed in toxicological studies of biopharmaceuticals. It is advised in the Japanese "Points to consider" document (Pharmaceutical Non-clinical Investigation Group, 2002; Nakazawa *et al.*, 2004) that the highest dose may be justified based on the observed plateau for the pharmacodynamic response without respect to toxicological changes (i.e., the maximum pharmacological dose). Other justifications for the highest dose include the emergence of a toxicological change, a multiple of anticipated clinical dose, or a maximum feasible dose. Because multiple different approaches are currently being used, additional scientific discussion may be necessary to establish the best method for setting the highest dose in a preclinical safety assessment study.

The use of select animal data to determine a starting dose for FIH has had little predictive value in some cases (Expert Scientific Group, 2006). For example, no toxicological changes were observed at the highest dose of TGN1412 in monkeys, which was determined to be the maximum feasible dose (Investigator's Brochure, 2005). Many reasons including species differences, insufficient preclinical data and lack of consideration for pharmacology information may have been involved in the failure to predict a safe starting dose TGN1412. The minimum anticipated biological effect level (MABEL) approach, recently proposed in a EMEA guideline (EMEA/CHMP/SWP/294648/2007, 2007), has been proposed as a better method to predict a safe starting dose for FIH from preclinical information. However, Ozaki *et al.* (2006) have argued that for FIH studies in Japan, such a conservative approach would slow down the development of biopharmaceuticals and that the conventional no observed adverse effect level (NOAEL) approach is more appropriate. Therefore, a balance between regulatory control and innovation is needed to deliver safe and effective new medicines to patients. Learning from implementation of the MABEL approach in the EMEA guideline and its effect on the safety and/or duration of clinical development should be considered during future ICH S6 discussions.

INDIVIDUAL STUDIES

1. Repeat dose toxicity studies

There seems to be disharmony among three regions regarding the regulatory requirement on the duration of non-rodent repeat dose toxicity studies (i.e., 6 months vs. 9 months vs. 12 months). Six-month studies are acceptable in Japan and the EU unless there is a specific concern for the investigational biopharmaceutical. Available data from approvals supports the position (Clarke *et al.*, 2007). Further scientific discussion is needed.

It is recommended in the ICH S6 guideline that immunogenicity should be measured and characterized in a repeat dose toxicity study. This information is helpful for the interpretation of toxicity study results, but it has little predictive value for immunogenicity in humans, as discussed in the ICH S6 guideline. Although the recommendation for immunogenicity testing is still useful, there does not appear to be a clear need for immunogenicity in all studies. It may be more efficient and informative

to conduct immunogenicity testing only when changes in biopharmaceutical plasma levels or toxicity potentially related to immunogenicity are important to the overall risk assessment.

2. Reproductive/developmental toxicity studies

Because the ICH S6 guideline allows flexibility in designing toxicity studies, a sponsor may consider conducting a modified reproductive/developmental toxicity study in rodents or rabbits even with mild immunogenicity. However, these conventional animal species may not be applicable if severe neutralizing antibody production occurs or if there is a lack of pharmacological response. In these cases, non-human primates (NHP) studies with the human product, studies in rodents with a homologue or studies in transgenic animals may be useful alternatives (JPMA and PMDA collaboration group, 2003; Nishimura, 2004; Evaluation Report). Among these alternate choices, NHP should be the first choice due to difficulties in interpreting data from homologues or transgenic animal as noted above. However, there are difficulties in using NHP for reproductive/developmental toxicity studies including low fertility, single fetus, relatively high abortion rate, long life cycle and seasonal reproduction with Rhesus monkeys. Furthermore, practical and ethical concerns impact the use of large number of NHPs per group (i.e., more than 12 females per group for Embryo Fetal Development Study). Therefore, historical data on NHP results from the testing facility is critical for the interpretation of results from these studies.

3. Safety Pharmacology

The ICH S7A guideline (2000) applies to both biopharmaceuticals and NCEs, but it is unclear from the scope in the ICH S7B guideline (2005) whether or not an *in vitro* cardiac channel assay, such as hERG and action potential duration (APD) assays, is required for biopharmaceuticals. Therefore, there seems to be some confusion among countries on the regulatory requirement. The Japanese "Points to consider" document (Pharmaceutical Non-clinical Investigation Group, 2002; Nakazawa *et al.*, 2004) suggests that such an *in vitro* study should not be applied for biopharmaceuticals because in contrast to NCEs, biopharmaceuticals are unlikely to interact with this cellular channel (Tristani-Firouzi *et al.*, 2001; Recanatini *et al.*, 2005).

Some new findings reported after the publication of Japanese "Points to consider" document suggest that the ion current through the hERG channel can be modified by agents that do not block the channel. It has been reported that some toxins have high affinity for and block the hERG channel (Zhang *et al.* 2003; Zhang *et al.*, 2007). The toxin binding site is located external to the channel and consists of a specific amino acid sequence. Although most biopharmaceuticals are unlikely to bind to such a specific toxin-binding site or produce a secondary blockade of hERG channel, this possibility cannot be ruled out. However, it is likely that these effects would be detected by *in vivo* electrocardiogram (ECG) evaluations. Therefore, it is recommended that if there is a signal indicating QTc effects in an *in vivo* study, the mechanism should be discussed in context with relevant scientific information and/or *in vitro* study data including the hERG assay. Furthermore, bioconjugates with an organic linker may have properties of both biopharmaceutical and NCE. If small fragments derived from a bioconjugate are a concern, they may have to be dealt with like a NCE. However, it may be difficult to identify, synthesize and examine all possible chemical fragments of a bioconjugate using *in vitro* studies. Therefore, the decision to conduct or not conduct an *in vitro* study should be made based on the results of an *in vivo* study in which both a parent bioconjugate and all fragments are tested as a whole for the potential of QTc prolongation. If a scientific explanation from existing information is possible for QTc prolongation observed in an *in vivo* study, additional *in vitro* study may not always be needed.

It has also been reported that tumor necrosis factor-α (TNF-α) consistently and reversibly decreased hERG current probably by stimulating superoxide anion (Wang *et al*, 2004). This is a secondary effect but not direct blockade of the hERG channel. Testing for these potential secondary effects of biopharmaceuticals is not expected.

4. Genotoxicity studies

Genotoxicity studies routinely conducted for NCEs are not needed for most biopharmaceuticals because of the failure of transmembrane penetration of biopharmaceuticals, due to their high molecular weight. As described in the previous section, genotoxicity studies with some bioconjugates may provide scientific value for the assessment of their genotoxicity risk (Gocke *et al.*, 1999). The decision to conduct genotoxicity studies and the experimental design should be scientifically justified. For example, if no degradation of a bioconjugate occurs or if there is a precedent for using a particular linker, genotoxicity studies may not be needed.

5. Carcinogenicity studies

According to ICH S6 guideline, a standard carcinogenicity assessment is not needed for most biopharmaceuticals. However, there may be a cause for concern

for some biopharmaceuticals when the clinical treatment duration, patient population and biological activities of biopharmaceuticals (e.g., growth factors and immunosuppressants) are considered. Nevertheless, the necessity of carcinogenicity assessment for growth factors and immunosuppressants has not yet been fully scientifically justified. For instance, it was recently reported that negative results with mouse and rat growth hormones were obtained in 2-year bioassays (Farris *et al.*, 2007). The rodent findings are consistent with existing clinical data suggesting no risk for tumors following human growth hormone treatment in patients (Allen *et al.*, 1997). Thus, the animal findings provide little additional value for the carcinogenicity risk assessment of biopharmaceuticals if there is enough human data with similar molecules. Besides human growth hormone, carcinogenicity assessments were conducted for insulin and its analogues, basic fibroblast growth factor, FSH and PTH (Advisory Committee Briefing Document, 2001; Hodsman, 2005; Barbehenn *et al.*, 2001; FDA Draft Guidance, 2000). The relevance of these studies to human risk has not been determined.

The concern associated with these growth factors or hormones is mitogenicity but not mutagenicity. Furthermore, in many cases, rodents are generally inappropriate for assessing biopharmaceuticals due to a lack of pharmacological response or neutralizing antibody production. Thus, a 2-year rodent bioassay should not be a regulatory expectation. Proliferative lesions noted by histopathological examination in a chronic toxicity study using a relevant animal could be an early indicator of potential carcinogenicity. For histopathological evaluation, techniques such as proliferative cell nucleic antigen (PCNA) or replicative DNA synthesis (RDS) is recommended in the chronic toxicity study. However, proliferative changes are clearly not sufficient to fully characterize the human risk, which can only be determined by clinical data. Two-step carcinogenicity testing may be an option if rodents are relevant species, while rodent studies using homologous proteins or surrogate antibodies, or the use of humanized mice (Bugelskil *et al.*, 2000), may be other choices. Besides those *in vivo* data, results of *in vitro* proliferation assay using a target cells may be useful for the risk assessment carcinogenicity. It is important to consider all options and to select an approach on a case-by-case basis using scientific justification for the selected evaluation.

CONCLUSION

Japanese experts from industry, regulatory bodies and academia recommend updating the ICH S6 guideline to reflect experience and knowledge accumulated over the last decade, although the "case-by-case" approach must be preserved as a basic principle. The major areas for the update are as follows: 1) Transgenic animals and homologous proteins could be an alternative in the case of no available relevant animal species; however, there are limitations with regard to the safety margin, validation, historical data, and physicochemical and pharmacological differences from the clinical candidate. Therefore, if a relevant animal species is available for the clinical candidate, a rodent study with a homologue usually is not needed. 2) Monkey reproductive/development toxicity studies are feasible and meet regulatory requirement, although there are some technical difficulties. 3) Most biopharmaceuticals cannot block potassium channels because they cannot penetrate inside the cell to block the channel. However, if QTc prolongation is observed in an *in vivo* study, an *in vitro* study including hERG should be considered. 4) Alternative approaches for the risk assessment of carcinogenicity (e.g. a chronic toxicity study with proliferative markers in a relevant animal) are useful and justified in many cases, since the concern for biopharmaceuticals is mitogenicity rather than mutagenicity. 5) Bioconjugates are a new category of ICH S6 and need specific considerations, while oligonucleotides should be out of scope.

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