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RESEARCH ARTICLE

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Imposex in marine gastropods may be caused by binding of organotins to retinoid X receptor

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Abstract Organotin compounds have been widely used as antifouling paints for ships and fishing nets since the 1960s and have thus been released into marine environments. Aquatic invertebrates, particularly marine gastropods, are extremely sensitive to organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) and undergo changes in sexual identity in response to exposure. This worldwide phenomenon is one of the worst consequences of pollution by man-made chemicals and has led to the ban of such compounds in antifouling paints in a number of countries, although organotin compounds still exist in the environment. So far, very low-concentrations of TBT or TPT have been shown to induce imposex (superimposition of male genitalia on female) in marine gastropods. Although the imposex induction mechanism has been controversial for many years, it was recently reported that TBT and TPT are potent and efficacious activators of retinoid X receptor (RXR), a member of the nuclear receptor superfamily. In this review, I discuss the involvement of RXR in the development of gastropod imposex.

Introduction

In their book "Our Stolen Future", Colborn et al. (1996) pointed out that a number of environmental chemicals affect hormonal systems and have adverse health effects on wildlife and probably on humans. Such chemicals are referred to as endocrine disruptors, and their effects have emerged as a major environmental issue. The nuclear receptors of intrinsic hormone systems are likely to be targets of endocrine disruptors, because their intrinsic ligands are fat-soluble and low-molecular-weight agents, as are the environmental pollutants. Many synthetic compounds, including the drug diethylstilbestrol (DES), dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCB), and alkylphenols, have been shown to bind nuclear receptors (Sohoni and Sumpter 1998; Blair et al. 2000; Nishihara et al. 2000; Gray et al. 2001). The effects of synthetic chemicals on sex hormone receptors such as the estrogen receptor (ER) and androgen receptor (AR) have attracted much attention, focusing on the reproductive failures observed in wildlife.

Organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s. Their release into the marine environment has resulted in pollution worldwide. Most marine gastropods in organotin-polluted areas have shown reproductive failure due to oviduct blockage by vas deferens formation, resulting in population decline or mass extinction (Bryan et al. 1986; ten Hallers-Tjabbes et al. 1994). This phenomenon is called "imposex" as an abbreviation of "imposed sexual organs", because male genital organs, such as the penis and vas deferens, are imposed upon female organs (Smith 1971). Approximately 150 species of imposex-affected gastropods have been found in the world (Fent 1996; Matthiessen et al. 1999). Gastropod imposex is reportedly induced by very low concentrations of TBT or TPT and is thought to be one of the mechanisms of endocrine disruption in wildlife (Smith 1971; Bryan et al. 1986, 1987, 1988; Gibbs and Bryan 1986; Gibbs et al. 1987; Axiak et al. 1995; Horiguchi et al. 1997b). Despite several hypotheses on the cause of imposex induction, such as aromatase inhibition, testosterone excretion-inhibition, functional disorder of the female cerebropleural ganglia, and involvement of amidated tetrapeptide Ala-Pro-Gly-Trp-NH2 (APGWamide) (Bettin et al. 1996; Ronis and

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Mason 1996; Oberdörster and McClellan-Green 2000, 2002), the detailed biochemical mechanism behind this phenomenon remains obscure.

It is well known that steroidal sex hormones such as 17β-estradiol (E_2) and 5α-dihydrotestosterone (DHT) exert important roles in physiological processes, including sexual development and reproduction in vertebrates. However, homologues of ER and AR have not been found in invertebrates (Escriva et al. 1997). Because gastropods are mollusks, they may not have functional receptors for androgen, suggesting that vertebrate-type sex hormones may not be involved in male sexual development in the gastropods. Recently, it was reported that TBT and TPT are high-affinity ligands for human retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR) γ (Kanayama et al. 2005). In addition, a functional homologue of RXR has been cloned from the rock shell (*Thais clavigera*) and the natural ligand of RXR, 9-*cis* retinoic acid, induces imposex in this species (Nishikawa et al. 2004). These reports suggest that the induction of imposex by organotin compounds may be mediated by RXR.

Differences in nuclear receptors between invertebrates and vertebrates

Nuclear receptors are structurally related proteins classified into a large superfamily that includes receptors for hydrophobic molecules such as steroid hormones (e.g., estrogens, androgens, progesterone, glucocorticoids, mineralocorticoids), retinoic acids (all-*trans* and 9-*cis* isomers), thyroid hormone, 1,25 $(OH)_2$ vitamin D_3, fatty acids. In addition to these receptors, the superfamily also contains a large number of so-called orphan nuclear receptors whose ligands do not exist or have not been identified (Giguére 1999). Nuclear receptors share a common structural organization with a highly conserved DNA-binding domain and a moderately well-conserved ligand-binding domain (LBD) (Fig. 1). Phylogenetic study and extensive polymerase chain reaction (PCR) surveys have revealed that nuclear receptor genes appeared very early on during metazoan evolution, but could not be found in fungi, plants, or unicellular eukaryotes (Escriva et al. 1997, 2000). By virtue of genome projects, we now know that *Homo sapiens*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, respectively, have 48, 21, and 220 kinds of nuclear receptor genes (Maglich et al. 2001). There is a striking difference between vertebrates and invertebrates with respect to their nuclear receptor sets. For instance, receptors for sex and adrenal steroid hormones have not been found in any fully sequenced invertebrate genomes. Although ER-like cDNA was reportedly isolated from the mollusk *Aplysia californica*, it could not bind to estrogens and was a constitutive activated transcription factor like the orphan nuclear receptors (Thornton et al. 2003). So far, functional steroid hormone receptors including AR, ER, progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR), have not been found in any invertebrate species (Escriva et al. 1997; Laudet 1997).

Reproductive abnormalities in wildlife can be associated with exposure to environmental pollutants capable of mimicking the action of sex hormones. In fact, there are many synthetic chemicals that have been shown to possess estrogenic activity by in vitro binding assay, reporter gene assay, or uterotrophic assay. The typical characteristic of chemicals having estrogenic activity is a phenol with a hydrophobic moiety at the para-position and without bulky groups at the ortho-position (Blair et al. 2000; Nishihara et al. 2000). Although these compounds may have adverse health effects in vertebrates (Colborn et al. 1996), they may not alter the function of the reproductive system through the medium of ER in invertebrates.

Imposex in marine gastropods

Among the variety of endocrine-disrupting events in marine invertebrates, imposex is one of the most documented. Imposex is induced by TBT at concentrations as low as 1 ng/L of tin (Sn) (Gibbs et al. 1987; Axiak et al. 1995) and is used extensively all over the world as a biomarker to monitor TBT pollution (Gibbs et al. 1987; ten Hallers-Tjabbes et al. 1994; Horiguchi et al. 1997a; Terlizzi et al. 1998, 2004). Not only TBT but also TPT has been shown to have a strong effect on the development of imposex in *T. clavigera* (Horiguchi et al. 1997b). So far, several hypotheses have been proposed to explain imposex induction. The first is that TBT increases androgen levels by inhibiting the enzyme activity that metabolizes testosterone. An aromatase enzyme complex is responsible for converting androgenic to estrogenic steroids. This enzyme complex consists of the microsomal CYP19 enzyme and the flavoprotein nicotinamide adenine dinucleotide phosphate reduced-form reductase. The latter is responsible for transferring reducing equivalents to CYP19 within the membrane of the endoplasmic reticulum. Bettin et al. (1996) reported that TBT increases androgen levels through inhibition of aromatase activity in marine neogastropods at relatively high doses. The TBT also inhibits the catalytic activity of human aromatase from transfected cells or a granulosa cell-like tumor cell line (Cooke 2002; Heidrich et al. 2001; Saitoh et al. 2001). However, it is doubtful whether the inhibitory effect of TBT on aromatase activity is a cause of the imposex, because the role of vertebrate sex steroids is unclear in invertebrates (LeBlanc et al. 1999). The second hypothesis is that TBT acts as a neurotoxin to abnormally release the peptide hormone termed penis morphogenic factor (PMF) (Féral and Le Gall 1983). The peptide hormone APGWamide has been proposed as the putative PMF, because injection of APGWamide significantly induces imposex in the mud snail *Ilyanassa obsoleta* (Oberdörster and McClellan-Green 2000,

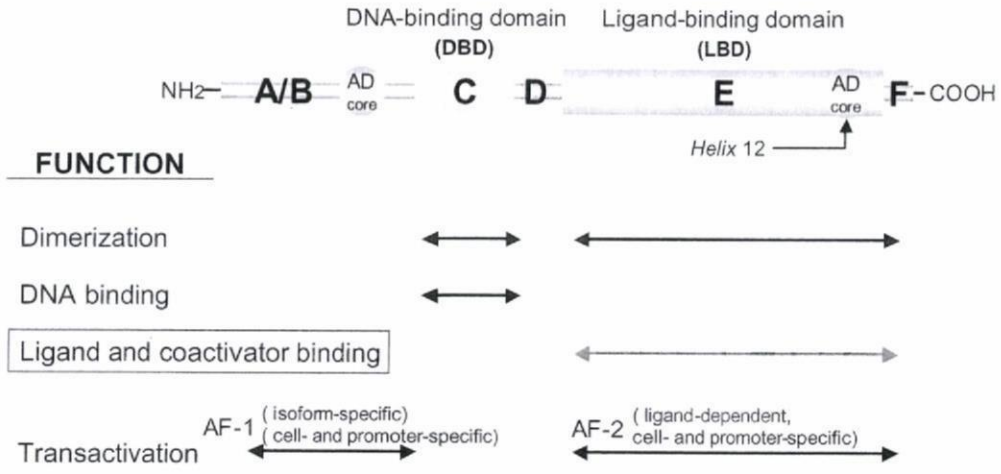

Fig. 1 Typical structure and functional domain of a nuclear receptor. Nuclear receptors are highly structurally related and share a common structural organization with a variable amino-terminal domain (**a**/**b**); a central, well-conserved DNA-binding domain (**c**); a non-conserved hinge domain (**d**); and a carboxyl-terminal, moderately conserved ligand binding domain (**e**). The ligand-independent transactivation function (**af**-1) is contained within the **a**/**b** region, and the ligand-dependent transactivation function (**af**-2) is within the **e** region

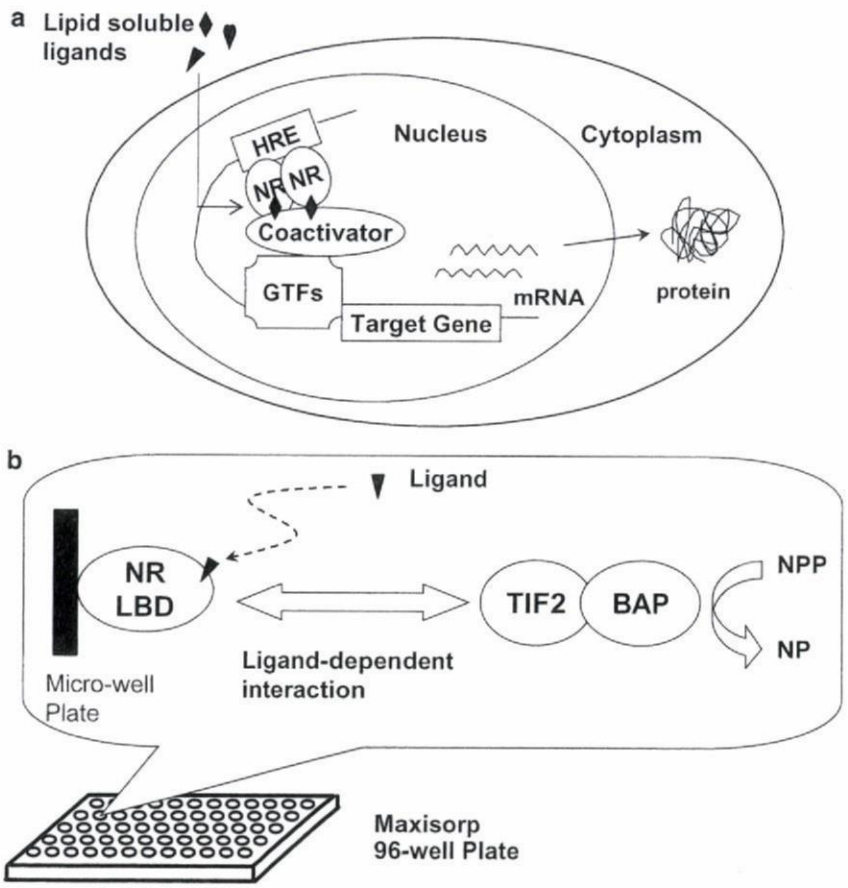

Fig. 2 **a** Nuclear receptors act as ligand-activated transcription factors by directly interacting with DNA-response elements of target genes as homodimers, heterodimers, or monomers. The effects of nuclear receptors on transcription are mediated through recruitment of co-regulators. Upon ligand binding, the receptors undergo a conformational change that allows the recruitment of coactivator complex. Recruitment of coactivator complex to the target promoter causes chromatin decomposition and transcriptional activation through interaction with general transcription factors (*GTFs*). **b** Principle of the screening method for nuclear receptor ligand. Nuclear receptor ligand-binding domain (*NRLBD*) is immobilized on the surface of a 96-well microplate. Coactivator TIF2 is prepared as a fusion protein with bacterial alkaline phosphatase (*BAP*). Test chemicals are added to the well with TIF2-BAP fusion protein. If the test chemical works as a ligand, it induces conformational change in NRLBD and recruits the TIF2-BAP on the plate surface. *p*-Nitrophenyl phosphoric acid (*NPP*) is used as a substrate for BAP. The BAP converts NPP to *p*-nitrophenol (*NP*), which appears *yellow*

2002). They proposed that PMF causes the development of male sex characteristics following an external stimulus such as TBT exposure. However, PMF cannot be the primary factor in the induction of imposex symptoms by TBT. There must be something other factor that directly interacts with TBT in the initial step of imposex induction.

Tributyltin and Triphenyltin as high-affinity ligands for nuclear receptors

Recently, Kanayama et al. (2005), by comprehensive screening of suspected endocrine disruptors versus human nuclear receptors, reported that TBT and TPT are high-affinity ligands for RXR and PPARγ. Their screening method was based on the ligand-dependent interaction between nuclear receptors and coactivators (Kanayama et al. 2003). In the initiation step of transcriptional activation, cognate ligands change the three-dimensional conformations of nuclear receptors (Bourguet et al. 1995; Renaud et al. 1995; Brzozowski et al. 1997; Agostini et al. 1998; Nolte et al. 1998). Next, a coactivator is exclusively recruited to its ligand-bound form of the receptor, but not to the ligand-free form (Fig. 2a). Kanayama et al. developed an in vitro detection method for ligand-dependent interaction between coactivator and nuclear receptors and applied it to the high-throughput screening (Fig. 2b). Using this system, they found that several suspected endocrine disruptors affected multiple nuclear receptors simultaneously. Among them, the effects of organotin compounds on RXR and PPARγ were most obvious. The agonistic effect of TBT on RXR was as strong as that of its endogenous ligand, 9-*cis* retinoic acid, and the effect of TPT on PPARγ was as strong as that of its well-known ligand, rosiglitazone. They also showed that TBT and TPT induced the transactivation function of RXR and PPARγ in mammalian culture cells (Kanayama et al. 2005). The dose range of TBT or TPT that induced transcriptional activation was 10–100 nM; this is almost pharmacologically relevant to the range reported to induce imposex in gastropods.

In mammals, PPARγ binds to DNA as a heterodimer with RXR and plays a central role in adipocyte gene expression and differentiation (Tontonoz et al. 1994). The PPARγ is abundantly expressed in adipocytes, and its ligands induce the efficient conversion of fibroblastic cells to adipocytes, as measured by induction of adipocyte-specific genes and lipid accumulation (Lehmann et al. 1995). In fact, TBT or TPT promotes differentiation of mouse preadipocyte 3T3-L1 cells to adipocytes (Kanayama et al. 2005). Therefore, organotin compounds may have adverse health effects on mammals by disturbing the endocrine processes mediated by the PPARγ/RXR pathway.

However, as I mentioned before, the composition of members of the nuclear receptor superfamily is quite different between vertebrates and invertebrates. The subgroup members of thyroid hormone receptor (TR), retinoic acid receptor (RAR), vitamin D receptor (VDR), and PPAR appear to have been late acquisitions during the evolution of the nuclear receptor superfamily (Escriva et al. 1997; Laudet 1997). Therefore, PPARγ might not be present in marine gastropods (Fig. 3). In contrast, RXR is special among the nuclear receptor superfamily. It is widely conserved in the evolutionary tree and its homologue, called ultraspiracle (USP), is found even in arthropods (Laudet 1997).

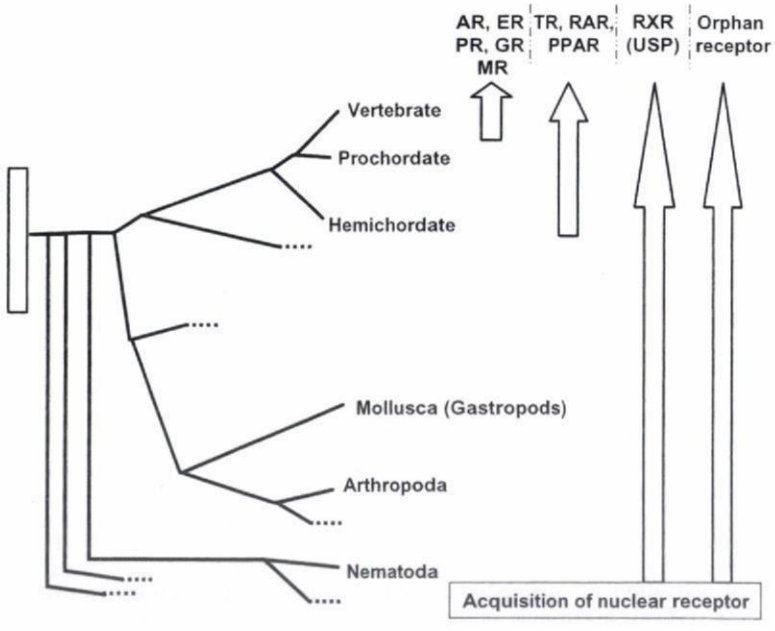

Fig. 3 Evolutionary tree and acquisition of nuclear receptors. Steroid hormone receptors (*AR* androgen receptor, *ER* estrogen receptor, *PR* progesterone receptor, *GR* glucocorticoid receptor, *MR* mineralocorticoid receptor) exist only in vertebrates. The subfamily of TR, RAR, and PPAR are present in vertebrates to hemichordates. In contrast, RXR or its homologue USP exist even in insects and nematodes

Characteristics of gastropod retinoid X receptor

Retinoid X receptor homologue has been cloned from *T. clavigera* (Nishikawa et al. 2004). Gastropod RXR has a DNA binding domain (DBD) composed of two C_2C_2-type zinc finger motifs and a putative LBD in the C-terminal region (Fig. 4a). The highest similarity with other species is in the DBD, where 85–90% of the amino acids residues are identical (Fig. 4b). The LBD of gastropod RXR also shows considerable similarity with that of vertebrate RXRs but has much less similarity with USP, the RXR homologue first found in *D. melanogaster*. Although RXR binds 9-*cis* retinoic acids in organisms ranging from cnidarians (*Tripedalia cystophora*) to vertebrates, USP from arthropods is unable to do so (Heyman et al. 1992; Mangelsdorf et al. 1992; Henrich and Brown 1995; Kostrouch et al. 1998). As expected by the similarity of a gastropod homologue to vertebrate RXR, the binding of gastropod RXR to 9-*cis* retinoic acid has been confirmed experimentally (Nishikawa et al. 2004). The dissociation constant in the binding of 9-*cis* retinoic acid to gastropod RXR is 15.2 nM, which is similar to the values reported for vertebrate RXRs (1–10 nM)

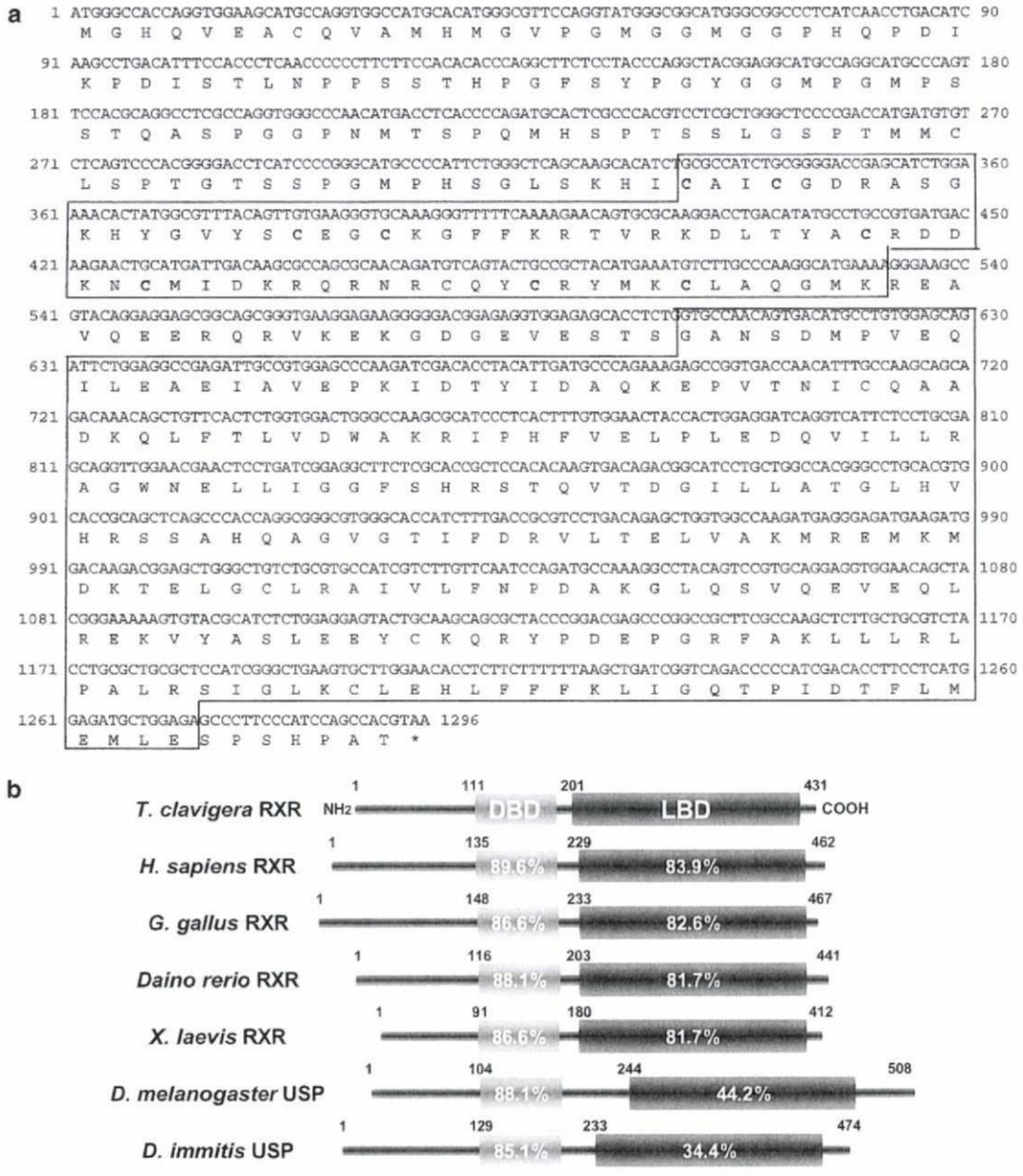

Fig. 4 **a** The entire coding sequence of gastropod RXR. The DNA and LBDs are *boxed*. The database accession number for the sequence is AY704160. **b** A schematic representation of RXR from various species is shown, along with the percentage of identical amino acid residues shared with those of gastropod RXR

Table 1 Inhibitory concentrations of 9-*cis* retinoic acid and organotin compounds in binding of radio-labeled 9-*cis* retinoic acid to human or gastropod RXR

Receptor	Compounds	IC_{50} (μM)
Human RXRα	9-*cis* retinoic acid	0.99
	Tributyltin	0.99
	Triphenyltin	0.85
Gastropod RXR	9-*cis* retinoic acid	0.81
	Tributyltin	8.16
	Triphenyltin	6.49

(Heyman et al. 1992). Gastropod RXR also binds to organotin compounds, even though the 50% inhibitory concentration (IC_{50}) values are larger than for 9-*cis* retinoic acid (Table 1).

Development of imposex in *Thais clavigera* by injection of 9-*cis* retinoic acid

Organotin compounds are potent and efficacious agonistic ligands of the vertebrate nuclear receptors RXR and PPARγ (Kanayama et al. 2005). It is worth noting that receptor activation is observed at nanomolar concentrations, whereas other mechanisms of toxicity (e.g., aromatase inhibition) occur in the micromolar range. Furthermore, there is a functional RXR homologue in gastropods that binds to both 9-*cis* retinoic acid and organotin compounds (Nishikawa et al. 2004). These facts suggest that gastropod imposex is mediated by RXR. Consistent with this prediction, Nishikawa et al. observed that 9-*cis* retinoic acid, like TPT, actually induces imposex in female gastropods (Nishikawa et al. 2004).

So far, certain neuropeptides have been considered as sex hormones in mollusks, as opposed to steroid hormones in vertebrates (LeBlanc et al. 1999). Oberdörster and McClellan-Green have demonstrated that only APGWamide, out of four neuropeptides, induces imposex in the mud snail (Oberdörster and McClellan-Green 2000). They put forward the model that the peptide hormones control the release of fat-soluble hormones, similar to the feedback control of the hypothalamic-pituitary axis in vertebrates (Oberdörster and McClellan-Green 2002). It is possible that APGWamide regulates the expression of some cytochrome P450 (CYP) that catalyzes the transformation of retinoids into active forms such as 9-*cis* retinoic acid.

Perspectives

This review has explored the concept that organotin-induced imposex in marine gastropods is mediated by nuclear receptors. This can be understood in terms of the molecules and mechanisms that regulate male sexual development in mollusks. Vertebrate-type steroid hormone receptors, including AR, are absent in invertebrates, suggesting that androgens may not act as

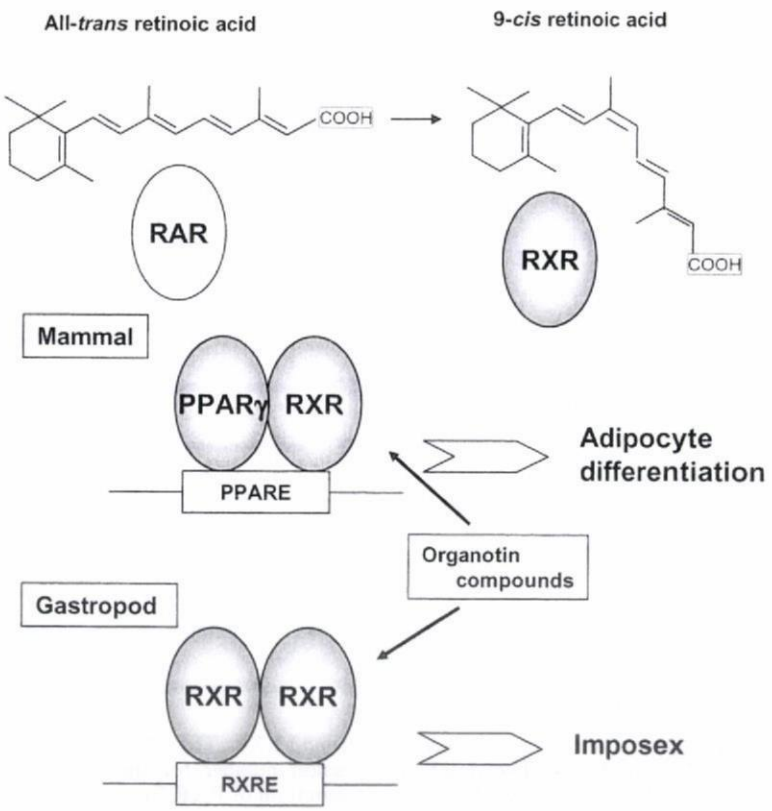

Fig. 5 Effects of organotin compounds in mammals and gastropods via RXR. In mammals, organotin compounds are ligands for both PPARγ and RXR and affect adipocyte differentiation. In gastropods, organotin compounds induce imposex by binding to RXR

male sex hormones. The TBT or TPT stimulates the development of the male genital tract in female gastropods. The TBT and TPT are high-affinity ligands for RXR. Gastropods have a functional homologue of RXR. The 9-*cis* retinoic acid, a natural ligand of RXR, significantly caused the development of imposex in female rock shells. These results suggest that RXR plays an important role in the induction, differentiation, and growth of male genital organs in female gastropods (Fig. 5).

In mammals, RXR is known to act both as a ligand-dependent transcription factor and as a common heterodimer partner for many non-steroid nuclear receptors (Mangelsdorf and Evans 1995). In the cases of some heterodimers, RXR is not activated by its own ligand (Mukherjee et al. 1997). In contrast, synthetic RXR-selective ligands activate RXR homodimer-dependent transcription (Boehm et al. 1995). Because 9-*cis* retinoic acid effectively induces imposex, RXR may function as a homodimer in gastropods. Meanwhile, we do not know whether gastropods inherently possess a pathway for the biosynthesis of retinoic acid. Therefore, we do not know whether 9-*cis* retinoic acid is a real hormone or whether similar derivatives are. We need to identify the active compound responsible for male sexual development in gastropods. Even in mammals, 9-*cis* retinoic acid is difficult to detect in vivo and its action remains obscure (Horton and Maden 1995). The study of retinoids in gastropod imposex may provide some insight into the physiological function of 9-*cis* retinoic acid.

Knock-out mice have provided important information on the physiological functions of these receptors. There are three subtypes of RXR in mammals. RXRα null mice die at embryonic days 12.5–16.5 and exhibit a hypoplastic ventricular myocardium as well as conotruncal and ocular abnormalities (Kastner et al. 1994; Sucov et al. 1994). Approximately 50% of RXRβ null mice die before or at birth, and the remaining male null mutants are sterile, owing to the aberrance of lipid metabolism in the Sertoli cells (Kastner et al. 1996). RXRγ null mice are viable and do not display any abnormalies (Krezel et al. 1996). Dysfunction study using RNAi or homologous recombination in gastropods will be needed to determine the role of RXR in the imposex development.

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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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Review

Points to consider on the non-clinical safety evaluation of anticancer drugs

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ABSTRACT — Since malignant tumors are life-threatening, the death rate from these diseases is high, and existing therapies have limited effectiveness, it is desired to provide new effective anticancer drugs to tumor patients sooner. However, there is no guideline regarding non-clinical safety studies on the development of anticancer drugs required for the first in human clinical trials and for the approval applications in Japan. Then, the Ministry of Health, Labour and Welfare (MHLW) established the collaboration group including regulatory, academic and industrial scientists to prepare the guideline on the non-clinical safety evaluation of anticancer drugs in 2004. As a guide for basic concept of non-clinical safety studies on anticancer drugs, the "Points to Consider" document was prepared by this group in 2007.

Key words: Points to consider, Non-clinical safety evaluation, Anticancer drugs, First in human clinical trial, Approval application

INTRODUCTION

For the test methods of non-clinical safety studies required for applications for approval to manufacture (import) drugs, various guidelines for test methods including "Guidelines for Toxicity Studies of Drugs" (Guideline for Toxicity Studies; MHW, 1989) have been published, and for the timing of non-clinical studies, "Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals" (M3 Guideline; MHW, 1998) specifies the timing. A wide variety of anticancer drugs have been developed, including those that directly or indirectly act on the cells causing cell death, primarily having what is called cytotoxicity, those that selectively block molecules involved in the signaling for cell growth in the new blood vessels required for proliferation of malignancy, and those that are highly selective because of their molecular-design such as tumor-specific antibodies. Since the existing non-clinical safety studies may not adequately examine high dose exposure over the clinical dosage range, or because of animal species specificity to the target molecules (i.e., some animal species do not respond to achieve the goal of the studies), or because of the severity of indicated disease, the therapeutic dosage and observed adverse reaction dose level may be close to each other, and for the social responsibility for providing patients with effective agents sooner, the type and duration of non-clinical safety studies are currently conducted on a case-by-case basis. However, because there is no basic concept, and because of these agents' clinical particularity that they are administered to patients from Phase I clinical trials, the study items and study contents may be inappropriate due to the varied concept of applicants, or the timing of study conduct may be inappropriate, and thus

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studies are not always conducted appropriately. Then, aiming for more appropriate safety evaluations are conduced at the appropriate time, as a guide for basic concept of non-clinical safety studies on anticancer drugs, "Points to consider on non-clinical safety evaluation of anticancer drugs" (Points to Consider) was established. With respect to cytotoxic anticancer drugs, "Q&A for toxicity studies conducted for clinical trails and approval applications for anticancer agents" (MHLW, 2004) was also published.

The objective of this "Points to Consider" is to minimize the risk of patients, and to promote the development of anticancer drugs by presenting the basic concept of non-clinical safety studies required prior to the initiation of Phase I clinical trials and approval applications for anticancer drugs in Japan.

However, it is basically difficult to specify a uniformed concept for all anticancer drugs. In addition, as the developmental stage of drugs advances, resulting in a great number of findings, conduct of new additional studies may be required. Therefore, so long as the results are beneficial to the clinical safety evaluation, the concept presented here is not always to be observed.

This "Points to Consider" should be applied to non-clinical safety evaluation of anticancer drugs developed for the purpose of providing cancer patients with some clinical benefits such as inhibition of progression and metastases of malignant tumor lesions, prolonged life and symptom relief. In the case of biologic anticancer drugs that have species-specific biological reactivity, the guideline entitled "Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals" (MHW, 2000) should also be taken into consideration when planning the appropriate safety evaluation studies.

Questions and answers for this "Points to Consider" are available in Supplemental Data.

BASIC CONCEPT OF NON-CLINICAL SAFETY STUDIES

General principles

The objectives of non-clinical safety evaluations are to 1) establishment of a safe initial dose level for the first human exposure, 2) clarification of the toxicological profile of a drug, e.g., identification of the target organs, estimation of the safety margin, and reversibility and 3) determining the proper endpoints to include for clinical adverse reaction monitoring. These objectives should be satisfied from the safety studies conducted in accordance with the "Guidelines for Toxicity Studies" (MHW, 1989) and "M3 Guideline" (MHW, 1998). However, because of the particularity of development of anticancer drugs, most often that Phase I clinical trials involve cancer patients; because the disease condition is progressive and fatal; because the treatment dose levels are often close to the adverse effect dose levels; because of the severity of disease; and because more effective new drugs are desired to be supplied to the patients sooner, the type and timing of safety studies of anticancer drugs may be different from those for other pharmaceuticals. This "Points to Consider" specifies the basic concept of the type and timing of safety studies in relation to the development of anticancer drugs, but it is difficult to specify a uniform concept because of the varied mechanism of actions for these drugs and the wide variety of indicated patient populations. Therefore, instead of adhering to this "Points to Consider", based on the previously notified "Guidelines for Toxicity Studies" (MHW, 1989), "Guidance on Genotoxicity Tests of Pharmaceuticals" (MHW, 1999), "Guidance on Carcinogenicity Tests of Pharmaceuticals" (MHW, 1999), "Safety Pharmacology Studies for Human Pharmaceuticals" (S7 Guideline; MHW, 2001), "Immunotoxicity Studies for Human Pharmaceuticals" (MHLW, 2006) and "M3 Guideline" (MHW, 1998) , and, at the same time, in light of the particular situations in which each anticancer drug in development is to be used, individually deciding the type and timing of safety studies from the scientific standpoint, the non-clinical safety should be appropriately evaluated.

Although this "Points to Consider" does not mention toxicokinetics, toxicokinetic assessments should be in compliance with "Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies" (MHW, 1996).

Non-clinical safety studies involving cancer patients, required prior to the initiation of Phase I clinical trials

1. Single dose toxicity studies

Single-dose toxicity studies should be conducted in two mammalian species as a rule (Notes 1 and 2). In at least one animal species, female and male animals should be studied, and approximate lethal dose should also be examined. The route of administration should be the clinical route of administration in humans as a rule. However, if the clinical route of administration in humans cannot deliver adequate exposure, the route of administration that can give a higher exposure level may be chosen.

2. Repeated dose toxicity studies

The schedule of administration (repeated administration or intermittent administration, intervals for intermit-

tent administration) for the repeated dose toxicity studies should be chosen from the standpoints of the pharmacological mechanism of action, the pharmacokinetics of the drug, the reversibility of toxic findings and the administration schedule and route in clinical trials under consideration.

The duration of administration should not always be in compliance with "M3 Guideline" (MHW, 1998). Since the primary objective of Phase I clinical trials is to determine a maximum tolerated dose (MTD) and dose limiting toxicity (DLT), if non-clinical repeated dose toxicity studies clearly demonstrated the presence or absence of toxicity by repeated dose administration, DLT and its marker, based on the results of toxicity studies conducted with shorter duration or a smaller number of cycles of administration, a clinical trial could be initiated. In the clinical trials, the duration or number of cycles of administration may be continuously increased according to the patient's response, and in this case, a new toxicity study would be unnecessary.

Repeated dose toxicity studies should be conducted in females and males of two animal species, a rodent and non-rodent. Determination of no observed adverse effect level (NOAEL) is not essential, but it is desirable to set the dose level, so that an adverse reaction marker and DLT could be determined. The accumulation and delayed toxicity should also be considered.

3. Genotoxicity studies

As a rule, genotoxicity studies are not mandatory.

4. Reproductive and developmental toxicity studies

As a rule, reproductive and developmental toxicity studies are not mandatory. However, the exclusion criteria should include pregnant female patients, nursing mother patients, and female patients who are possibly pregnant. If the repeated dose toxicity studies suggested the effect on male reproductive organs, male patients should be instructed to practice strict contraception. Even when reproductive and developmental toxicity studies are not conducted, female patients of childbearing potential may be included in a clinical trial if strict contraception is observed (Note 3).

5. Local tolerance studies

When the clinical route of administration is parenteral, a conduct of local tolerance studies should be considered. However, a detailed examination such as a histopathological examination on the administration site in single dose or repeated dose toxicity studies can substitute local tolerance studies.

6. Safety pharmacology studies

Comply with the "S7 Guideline" (MHW, 2001).

Non-clinical safety studies required prior to the initiation of Phase I clinical trials conducted in healthy adults

Comply with the "M3 Guideline" (MHW, 1998).

Non-clinical safety studies required prior to the approval application

1. Repeated dose toxicity studies

Repeated dose toxicity studies should be conducted in males and females of two animal species, rodent and non-rodent. However, when an anticancer drug applicable to only male or female patients demonstrated no apparent sex difference in short-term repeated dose toxicity studies conducted in two animal species, and the pharmacokinetics suggested no sex difference, long-term repeated dose toxicity studies may be conducted in the sex that is subject to treatment in clinical practice. The administration (daily administration or intermittent administration, intervals for intermittent administration) for the repeated dose toxicity studies should be chosen, taking the pharmacological, pharmacokinetic characteristics of the drug, the reversibility of toxic findings, and administration in clinical trials into consideration. The duration of administration should not be more than 6 months in rodents and 9 months in non-rodents as a rule, but considering the toxicological characteristics of the anticancer drug and duration of treatment in clinical practice, setting a more appropriate duration may be desirable. The studies should be designed so as to either one of the studies can examine the reversibility.

2. Genotoxicity studies

As a rule, genotoxicity studies should be required. However, when genotoxicity is predicted from the mechanism of action and so on, the standard battery of genotoxicity studies may be partially omitted.

3. Carcinogenicity studies

Normally, carcinogenicity studies are not required. See "Guidance on genotoxicity tests of pharmaceuticals" (MHW, 1999) for details.

4. Reproductive and developmental toxicity studies

As a rule, reproductive and developmental toxicity

studies are required. However, if the mechanism of action has been suggested to have an embryonic lethality or teratogenicity, the reproductive and developmental toxicity studies may not be required for an approval application. In these cases, pregnant female patients, nursing mother patients, and female patients who are possibly pregnant are contraindicated, and female patients of childbearing potential and male patients are instructed to practice strict contraception.

When the reproductive and developmental toxicity, especially teratogenicity is to be examined, because of the nature of the drug such as potent maternal toxicity or embryonic lethality, the duration of administration or intervals varied from those specified in "Guidelines for Toxicity Studies" (MHW, 1989) must be considered. In this case, the rational for it requires scientific explanations.

5. Immunotoxicity studies

Comply with the "Immunotoxicity Studies for Human Pharmaceuticals" (MHLW, 2006).

6. Safety pharmacology studies

As a rule, safety pharmacological studies are required. See "S7 Guideline" (MHW, 2001) for details.

Notes

Note 1: In setting the initial dose for the Phase I clinical trials, if there is a scientific rationale for administration to rodents, a study in non-rodents is not always required.

Note 2: "Guideline for Clinical Evaluation of Anti-Malignant Tumor Agents" (MHLW, 2005) specifies that the initial dose of Phase I clinical trials should be 1/10 of the 10% lethal dose (LD_{10}) in mice presented by mg/m^2 as a rule, and if this dose demonstrated toxicity in other animal species, based on the animal species showing maximum sensitivity, the initial dose should be lower than a minimum dose showing no irreversibility. From these above, when a drug is to be administered to humans for the first time in Japan, studies should be designed so as to establish a toxic dose such as LD_{10} in mice, which is to be a rationale for deciding the initial dose. However, when reliable foreign Phase I clinical trial results or treatment results in humans are available, the initial dose for Phase I clinical trials can be chosen from those other than the results of non-clinical safety studies.

Note 3: Female patients of childbearing potential should be confirmed the possibility of pregnancy, and male patients and female patients of childbearing potential should be instructed to practice contraception for an appropriate period after the end of treatment period.

Supplemental Data:

Supplemental data are available at the on-line version of this article (http://www.jtoxsci.org).

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Future alternatives in "3Rs": Learning from history

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Abstract
A large decrease in the number of experimental animals utilized in testing and research was reported in the last decade (Surveyed by Expt'l Animal Soc.[1]). For rats, the numbers used in experiments in Japan were 2.09 million in 1995, 1.53 million in 1998, and 1.24 million in 2001. Thus, there was a 40% decrease in the number of rats used from 1995 to 2001. For mice, a larger decrease (58%) was also observed, from 6.68 million in 1995 to 2.80 million in 2001. These decreases were clearly due not only to the development of 3Rs (i.e., Reduction, Refinement, and Replacement of animal use) in alternative research, but also to marked changes in the focus of experimental animal biology. In the academia, animal experiments using wild-type mice have decreased in number to a large extent relative to those using genetically modified mice because of the mechanistically much reliable outcomes obtained by genetically modified mice than those from wild-type animals. Yet, biological safety studies for pharmaceutical development as well as industrial chemical safety studies utilize conventional toxicological bioassays.

Keywords: 3Rs, Claude Bernard, Bruce N. Ames, Patric O. Brown

Introduction

Historically, three scientists are recognized in relation to the history of experimental animal use; the first, the initiator of experimental animal research; the second, the first contributor to the marked reduction of the number of experimental animals used; and the third, a potential contributor, who invented an ultimate method for reducing the number of animals for future research, the gene chip technology. The use of animals in experimental studies was initiated by Claude Bernard (1813-1878), originally who was trying to put an end to human vivisections common at that time; thus, he came to be regarded as "the devil of experimental animals." The most remarkable contribution to reducing the number of experimental animals used was made by Bruce N. Ames, who rescued innumerable animals that might have been used for genotoxic carcinogenicity studies. Another contribution may be attributed to Patrick O. Brown, who invented transcriptomics, which can be used to elucidate the underlying mechanistic background of phenotypes of experimental animals; the method is considered to have eventually led to the minimization of experimental animal use. Consequently, the most essential and powerful driving force for future alternatives may be minding the 3Rs but also the promotion of basic sciences and technologies.

1. Claude Bernard – An initiator of animal experiments

Claude Bernard (1813-1878)

Bernard was born in the village of Sain-Julien in 1813, and went to Paris at the age of twenty-one. As reported, he first wanted to be a play wright, but took up medical studies on the advice of a literary person. He learned medical science from the famous Françoise Magendie, and earned his PhD after pursuing the study of gastric acids. He was appointed as Magendie's deputy professor at the college in 1847, and made seminal discoveries such as those of hepatic glycogen, vasomotor neurons, and curare