Current Status of Conducting Alternative Testing to Mammalian Toxicity Studies in the Japan Pharmaceutical Manufacturers Association

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Abstract

To measure the use of alternatives to *in vivo* mammalian toxicity studies during drug research and development, the Japan Pharmaceutical Manufacturers Association (JPMA) distributed a questionnaire to its 99 member companies, of which 85 responded and 50 (59%) of them had some experiences in using alternatives to *in vivo* mammalian toxicity studies.

The results show alternative methods have been used foremost in hepatic toxicity testing, followed by teratogenicity, renal toxicity, eye irritation, skin irritation, cardiac toxicity, bone marrow toxicity and testicular toxicity tests. The alternative methods, regardless of category, provided screening and mechanistic analyses of the results of toxicity. Alternative test systems included isolated cells, primary culture cells, and established cell lines of mammalian species. For hepatic toxicity testing, primary culture cells were most commonly used. Other materials, such as perfused or sliced organs, were also used. For cardiac toxicity and neurotoxicity testing, primary culture cells were most popular; established cell lines were used in renal toxicity. Whole embryonic culture was mostly used in the primary culture alternative method for teratogenicity studies. The results also showed that animal welfare and cost savings were of minor importance in the opinion of most companies that responded.

Introduction

Most toxicological studies performed for pharmaceuticals before marketing are whole body (*in vivo*) animal studies using laboratory animals. These studies are required by the regulatory authorities in any developed countries in order to assess the potential adverse effects in humans. On the other hand, the concept of animal welfare and protection has recently been accepted widely, up to regulatory authorities (e.g., USDA, 1989 and 1991). Substantial goal of "3 Rs" i.e., refinement, reduction and replace-

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ment, made clear what to be done. However, the manufacturers tend to stick to the traditional animal studies, as these toxicity tests have been used and are presently required for the pharmaceuticals, in spite of some insufficiencies of the animal toxicological testing such as the species difference between animals and humans. *In vitro* testing provide new aspects in the safety assessment, for screening, mechanistic investigation and economical impacts, and thus, the manufacturers especially the pharmaceutical companies are becoming aware of the use of *in vitro* tests.

There are many reports on the status of use of alternatives to *in vivo* toxicological studies in industries of Europe and US (Balls and Fentem, 1992, Rozenkranz et al., 1992, Zucco, 1992, Fentem et al., 1992, Fry 1993). There has been no such report so far in Japan.

The present paper is concerned with the current status of the use of alternative testing in pharmaceutical industries in Japan, reporting the survey conducted in 1995 by the General Toxicology Working Croup (GTW) of the Japan Pharmaceutical Manufacturers Association (JPMA).

Methods

Alternative testing in this present questionnaire survey was defined as an *in vitro* study designed as an alternative to an *in vivo* animal study described in the Ministry of Health and Welfare (MHW,1989) and/or OECD (1981) guidelines for toxicity study. These were classified into several categories such as hepatotoxicity, renal toxicity, cardiotoxicity, neurotoxicity, sensitization and immunotoxicity, reproductive organ toxicity, teratogenicity, carcinogenicity, genotoxicity (excluding generally established *in vitro* studies), local irritation test (eyes, skin and others), drug-dependency and acute toxicity.

The questionnaire asked JPMA member companies to select one of the following statements about their experience in conducting an alternative test : [A] usually conduct alternative tests; [B] sometimes conduct, but do not routinely conduct any alternative tests; and [C] no experience with alternative tests. In cases of [A] or [B], the questionnaire asked the member companies to state for conducting the validity of alternative testing.

The questionnaire also asked the member companies to state their purpose for adopting the alternative test and which test systems they used, e.g., organ perfusion, sliced tissues, isolated cell lines, subcellular fractions, non-biological materials or other systems.

Results

Overview of the survey

The questionnaire was sent to 99 member companies, of which 85 companies responded. Table 1 shows the current status of alternative toxicology tests in JPMA. Forty (47%) out of 85 companies responded to the questionnaire with [A] for one or more categorized toxicity studies, meaning they were running one or more kinds of alternative testing routinely. Ten companies answered with [B], which means they have some experience but may not conduct such tests routinely. The other respondents had no experience with any alternative testing. Table 1 indicates the current status of alternative testing for 23 categories of toxicity. Results analyzed by the toxicity category revealed that hepatic toxicity had the highest proportion of alternative testing followed by teratogenicity, renal toxicity, eye irritation, skin irritation, cardiac toxicity, bone marrow toxicity, neurotoxicity, vessel irritation, testicular toxicity and carcinogenicity.

Current status of alternative testing by test category

The reactions the companies gave for adopting alternative testing, and the test systems adopted or tried are shown in Tables 2 and 3. These data include those companies that have any methods, are trying to establish new methods, or are planning new methods.

1. Acute Toxicity

Only one company was currently using some methods. The alternative test under development uses established cell lines to do some screening

Category of the test	Total*	Conduct *in vitro* tests		Others
		Usually	Sometimes	(*in vivo* tests only)
Adrenal toxicity	5	0	2	3
Antigenicity/ Sensitization	82	0	3	79
Bone marrow toxicity	7	1	4	2
Carcinogenicity	78	1	2	75
Cardiovascular toxicity	80	0	7	73
Eye irritation	69	2	8	59
Gastro-intestinal toxicity	2	0	1	1
Hepatic toxicity	82	6	19	57
Immunotoxicity	81	1	0	80
Mucous membrane irritation	3	0	1	2
Muscle irritation	3	1	1	1
Neurotoxicity	81	0	4	77
Ocular toxicity	3	1	2	0
Pituitary toxicity	1	1	0	0
Prostate toxicity	1	0	1	1
Renal toxicity	82	3	7	72
Skin irritation	68	1	8	59
Teratogenicity	84	3	12	69
Testicular toxicity	12	0	3	9
Thyroid toxicity	3	0	2	1
Vessel irritation	6	1	3	2

Table 1. Prevalence of *in vitro* toxicity testing in the JPMA companies
Total : Number of companies that answered the questions in total
Usually : Number of companies conducting some tests usually as routine work
Sometimes : Number of companies conducting some tests sometimes on a case-by-case basis
Others : Number of experience with alternative tests (*in vivo* tests only)
*The questionnaire was sent to 99 member companies of JPMA, and the maximum of 85 companies responded to the survey.

tests. The majority of companies is not considering any alternative testing in this field. There have been established and authorized alternative methods as for the acute toxicity test applied to chemicals in general for the classification purpose. They may not be considered applicable to the pharmaceuticals.

2. Adrenal toxicity

Two companies have established test systems, and implemented them on a “case-by-case” basis. The purposes are for mechanistic analyses of toxicities and for screening. The main test system was the primary cell culture of various mammals (rat, hamster, monkey, cattle, rabbit, dog, guinea pig). The effects were evaluated by measuring hormone secretion such as corticoid, the uptake abilities of neutral red (NR), release of the ADP or LDH content, or cell death (Higashijima et al., 1987; Hornsby et al., 1974; Vernetti et al., 1993).

3. Antigenicity and sensitization

Only a few companies have actually done this testing. The purposes of the testing were screening and mechanistic analyses of the toxicities. Some companies did the tests to improve the predictability of toxicity for human extrapolation. The test systems used were isolated cells (rat mast cells and human peripheral lymphocytes from blood). The amount of free histamine from rat mast cells was measured to check

Category of tests	Screening	Mechanistic	Animal Welfare	Cost Reduction	Others
Adrenal toxicity	2	5	0	0	0
Bone marrow toxicity	5	5	0	0	0
Cardiovascular toxicity	4	6	1	1	0
Eye irritation	12	4	6	3	1
Hepatic toxicity	28	22	5	7	1
Immunotoxicity	3	3	0	0	1
Neurotoxicity	2	5	0	0	0
Renal toxicity	8	9	1	2	0
Skin irritation	11	0	5	3	0
Teratogenicity	8	15	1	0	2
Testicular toxicity	1	4	0	0	0

Table 2. Purpose of major alternative research to animal toxicity testing*
*Number reflects the total of those companies using, planning or developing an *in vitro* test.

the antigenicity. Also, the number of cells producing specific antibodies is measured to indicate an induction of an immune response after a test material is added to human lymphocytes isolated from peripheral blood.

4. Bone Marrow Toxicity

One company was doing this test routinely. A test method has been established by four companies. The purposes of the tests were mainly mechanistic analyses of toxicities. The main test system was primary culture cells, followed by isolated cells (Du et al., 1992; Letza et al., 1988). The cells originated from bone marrow cells of animals and erythrocytes. The effects of hemopoietic cells are checked by the colony generation method. The cell division ability was checked by adding ^{3}H-thymidine to cultured cells after the cells were treated with test materials. Hemolysis of erythrocytes was also checked as a marker.

5. Carcinogenicity

One company was doing *in vitro* carcinogenicity test routinely for the screening purpose. Though a test method had been established, it was carried out only on a case-by-case basis by two companies. Two companies conducted the tests for screening and clarification of onset mechanisms.

Most common test systems were established cell lines, examining the effects on gap binding, the phenotypic transformation test (Oyamada and Yamasaki, 1989), and checking replicative DNA synthesis (RDS) using perfused organs.

6. Cardiac toxicity

Ten companies had experienced alternative testing for cardiac toxicity. The purpose in adopting the tests was mostly mechanistic analyses of toxicities and screening. The main test system used was primary cell culture. Evaluation was done by measuring enzyme activities (e.g., GOT, CPK) in cells, pulse changes, and checking morphological changes. In the perfused heart system, intra-cardiac pressure or ECG was measured.

7. Drug dependency liability

There was no company doing any alternative testing for dependency. A few alternative methods seem to have been developed in this field (Katsura et al., 1994).

8. Eye irritation

The tests were routinely done by two companies, and test methods had been established but not routinely done by eight companies. The test system used was isolated rabbit corneal epithelium (Minami et al., 1993 Sakemi et al., 1991;

Category of tests	A	B	C	D	E	F	G	H	I	J
Adrenal toxicity			1	4	1					
Bone marrow toxicity			2	4			0			
Carcinogenicity	1				4					
Cardiovascular toxicity	1		1	8						
Eye irritation				7	6		1		2	
Hepatic toxicity	3	3	4	29	2					
Immunotoxicity			2	2						
Neurotoxicity	1			5	1		2			
Renal toxicity.		1	1	2	8					
Skin irritation				3	4				4	
Teratogenicity				4	2		4	2		18
Testicular toxicity	1			5	1		1			

Table 3. Test systems* of major alternative tests to *in vivo* toxicity tests
A: perfused organ, B: slice organ, C: isolated cell, D: primary cell culture, E: cell line, F: sub-cellular fraction, G: organ culture, H: non-mammalian, I: non-biological lines, J: whole embryo culture
*Number of companies that answered the question include those which did the tests, are trying to establish a new method, or are planning a new test.

Watanabe et al., 1989).

9. Gastrointestinal Toxicity

One company was doing a test for mechanistic analyses of toxicities, using perfused organs. Gastric surface mucous cell lines from mice were established by Sugiyama et al., 1993.

10. Hepatic toxicity

Alternative testing methods were most commonly adopted in the test for hepatic toxicity and some correlation between *in vivo* and *in vitro* test results have been shown.

The most common purpose for adopting the test was for screening in exploratory research, followed in order by mechanistic analyses of toxicities, cost saving and animal welfare (Table 2). The test system used was most often based on primary cell culture (Deboyser et al., 1989, Viau et al., 1983, Ratanasavanh et al., 1988, Nakamura et al., 1985, Jurima-Romet et al., 1991, Boelsterli et al., 1987, Borenfreund and Puerner 1985), followed by fresh isolated cell suspension (e.g., Nakagawa et al., 1992), perfused organs (Shiota et al., 1985) or sliced organs (Smith et al., 1985, Barr et al., 1991, Wright and Paine 1992), and established cell lines (Viau et al., 1993). A primary cell cultures used were of rat origin in most cases, but also came from dogs, rabbits, and humans in some cases. Toxicity in the primary culture system was determined by assessing various enzyme activities (e.g., LDH, GOT, GPT, CPK) in more than half of the companies. Morphological observation of cells, the damage to mitochondria examined by the MTT assay, and quantitative analyses of lipid lines and lysosome damage by the neutral-red uptake assay were done in a small number of companies. Other test items included albumin synthesis, phagocytosis of Kupper cells, cytochrome P-450 measurements, peroxisome proliferation of a compound, and interaction with other drugs. Organ toxicity was assessed by measuring leaked enzyme activities and morphological changes in other test systems. Bile excretion, oxygen consumption, and drug concentration were also measured in perfused organ systems. Damage to organelles was evaluated in established cell lines. Some companies used liver slices of dogs and monkeys in addition to those of rats (Dogteron, 1993).

11. Immunotoxicity

No company had experienced doing *in vitro* immunotoxicity test. Only one company was doing *ex vivo* immunotoxicity tests routinely

(Luster et al., 1988, 1992). The principal uses of the tests were mechanistic analyses of toxicities and screening. The test systems used were primary cell culture and isolated cells, which originated from the spleen of mice and rats and lymphocytes of peripheral blood in rats or humans (Wood et al., 1992).

12. Mucous membrane irritation

One company was doing alternative testing using urinary bladder membrane as a screening prior to *in vivo* irritation studies.

13. Muscle irritation

Alternative testing in this field had been conducted by two companies. A primary cell culture was used in mechanistic analyses of toxicities, and CPK or histopathologic examinations were used by one company to evaluate the damage to the muscle (Kato et al., 1992). At another company, primary cell culture or established cell lines had been established for the purposes of screening, mechanistic analyses of toxicities, and animal welfare. The tests were not done routinely by then.

14. Neurotoxicity

Four companies had experienced alternative testing for neurotoxicity. The main uses of the tests were mechanistic analyses of toxicities and screening. The test system used was mainly primary cell culture. Various indicators such as GABA receptor binding, cell death rate, and enzyme activity were measured (Ogura and Kudo, 1988; Sher, 1991). Other systems included cultured organs with nerve projections as an indicator and a nerve-muscle combination specimen to observe muscle constriction in response to nerve stimulation.

15. Ocular toxicity

Three companies which have established test systems for screening and mechanistic analyses of toxicities responded. The test systems used were included to measure GSH levels and ATP ase activity in the lens, to examine direct effects of test materials, and cultured rat lens to measure the proliferation rate of epithelial cells (Xu et al., 1992).

16. Pancreatic Toxicity

One company was currently trying to establish a test method to check the secretion of insulin from the islet of Langerhans using the primary cell culture of rats. This was being done to evaluate the side effects in a similar type of compound to those with pancreatic toxicity (Sako et al., 1986).

17. Phototoxicity

Two companies which have established test systems for screening responded. Established cell lines were used. There are some *in vitro* phototoxicity methods such as neutral-red uptake using BALB/3T3 and NB1RGB, red blood cell photohemolysis, hemoglobin photooxidation and measurement of singlet oxygen (Arakane et al., 1996, Tabuchi et al., 1995).

18. Pituitary Toxicity

One company was doing tests routinely for mechanistic analyses of toxicities. Membrane current was measured by a patch clamp method, and hormones were measured using established cell lines (Oxford and Tes, 1993).

19. Prostate Toxicity

A test method has been established by just one company for mechanistic analyses of toxicities. The binding of test material to testosterone receptors was measured by using isolated cell fraction (Hosokawa et al., 1993).

20. Renal toxicity

Ten companies had experience in alternative testing for renal toxicity. The primary purpose of adopting alternative testing for renal toxicity was the greater part of screening and mechanistic analyses of toxicities. The test systems used were mostly established cell lines. Various kinds of enzyme activity (e.g. LDH, NAG) were measured in rat-derived NRK52E cells or swine-derived LLCPK1 cells in testing with established cell lines. The indicators were mostly cell morphology and enzyme activity. Cell death, cell proliferation and lipid content were also mea-

sured by some workers (Fukunishi et al., 1989, Williams et al., 1988, Boogaard et al., 1989, Bruggeman et al., 1992).

21. Skin irritation

The test was routinely done or a method had been established for one company each. Eight companies were experimenting with alternative methods.

The main purpose of testing was screening, followed by mechanistic analyses of toxicities. Companies routinely using *in vitro* tests mentioned animal welfare and cost reduction as compelling reasons for implementing the methods. The test systems for both eye and skin irritation were primary culture cells and established cell lines. Some nonbiological test kits were also used (Sasaki et al., 1992). Cultured organ systems were also used for eye irritation.

22. Teratogenicity

This was the second most popular field for which the *in vitro* testing was done. Fifteen companies had experience with in vitro teratogenicity testing. The main uses of the tests were mechanistic analyses of teratogenicity and screening. Cost saving was not a factor for any company responding. The most common test system was a whole embryo culture, obtained mostly from rats. The micro-mass culture method is used to check the effects on differentiation in limb buds of central nervous tissue removed from rat embryos. Systems using mammalian cells included primary cell culture and established cell lines. Those used were strains for measuring proteoglycan synthesis ability in limb buds, checking the effects of lectin adherence ability in Ehrlich cells, and evaluating proliferation of palate cells of human embryos (Peters and Piersma, 1990; Gray and Kimber, 1989). Chick embryo was used by 7% of the companies instead of the mammalian species, but this is still on a trial basis.

In the whole embryo culture systems (Ninomiya et al., 1993, Brown and Fabro, 1985, Klug et al., 1992, Uphill et al., 1990, Peters and Piersma, 1990), fetuses of mice, rats, or rabbits are removed between Gestation Days 8 and 12 and are cultured in 100% plasma. Lethality, growth suppression, and teratogenicity of the cultured fetuses exposed to test materials were measured. It was not completely alternative testing, because the duration of the culture was limited, and the viable period was short. However, it was often used to clarify mechanisms of teratogenicity in relation to "*in vivo*" studies. The survey results also indicated the main reason for the test was mechanistic analyses. The disadvantages of this test method include financial impact and ethical issues. In addition to culture media, 100% plasma requires many animals from which to harvest plasma, which also affects study cost. If an artificial culture medium is developed in the future, this issue might be resolved.

In the micro-mass culture method (Friedman, 1987, Shiota et al., 1990ab, Uphill, 1990), using mouse palate or rat embryos, the primordial four limbs and tissue of mesencephalon from the embryos are removed and cultured to evaluate the effects of exposure to test materials on development and differentiation. Generally speaking, there are some problems in the correlation of these results with " *in vivo*" study results. Evaluation by using multiple strains may be necessary. The uses of this test were mechanistic analyses and screening. The method used in mammalian cell culture (Pratt and Wills, 1985) is the same as in the micro-mass culture method; that is, tissues are removed, cells are isolated and cultured, and biochemical parameters in cell toxicity and cell differentiation are measured. The main use of this test was for screening rather than mechanistic analyses, according to the survey. Some responses raised questions on the correlation to *in vivo* study results.

23. Testicular toxicity

Three companies thought some alternative testing in this field was necessary. The purposes of testing were mainly mechanistic analyses of toxicities and screening. The test systems used were mainly primary cell culture, followed by isolated cells, organ slices, and cultured organs (Foster et al., 1987, Kazawa et al., 1990, Noguchi et al., 1987). Evaluation indicators were test-

osterone measurements, the measurement of functional markers on Sertoli cells, and cell death.

24. Thyroid Toxicity

Two companies formerly did the tests. They no longer do the tests for the following reasons.

In the established cell line systems, FRTL-5 cells were used to measured the inhibition rate of test material on $Na^{125}I$ uptake into cells. The data were highly variable, and the correlation with *in vivo* results was questionable. The system requires specially designated facilities for radiolabeled material, which adds to the problem. In primary cell culture systems (Ambesi-Impiobato et al.,1980), the method was examined to assess whether isolated and incubated thyroid follicles or follicular cells could be used to observe the effects of a test material (Tamaki et al., 1984).

25. Vascular irritation

Four companies were doing alternative testing in this field for mechanistic analyses of toxicity, screening and cost savings. The test systems used endoepithelial cells or smooth muscle cells of blood vessels. Quantitation of FITC-marked albumin or enzyme activities or a morphological technique was used for evaluation (Hoorn et al., 1993).

Consideration on validity of alternative testing

Reasoning for adopting alternative tests were asked. Forty-two out of 49 companies responded to the questionnaire with usually conduct validation or sometimes conduct. Five had used alternative tests with showed no reasoning validations. The responses have been classified roughly as follows of the companies : 32% used a comparison with a positive control substance as the reasoning 22% a correlation with *in vivo* study results; 15% a comparison with background data (including comparison with a negative control substance); 13% comparative studies among facilities (interlaboratory validation studies); 13% a comparison with literature data ; and 5% reproducibility of the test results.

Most companies with alternative testing experience responded that some kind of validation had been achieved (Zucco 1992).

Future Plans for Alternative Testing

Fifty-nine companies among JPMA members gave their opinions in response to the question about the future of adopting alternative testing. Comments were as follows : 73% plan to accept them; 22% have no plan to accept them; and 5% will use them on a case-by-case basis.

The purposes of the testing, as conveyed by those who would accept the testing with certain conditions, were shared equally between mechanistic analyses and screening.

The actual toxicity fields for which alternative testing may be used in the future were local irritation, hepatic toxicity, testicular toxicity, teratogenicity, renal toxicity, cardiac toxicity, bone marrow toxicity, and carcinogenicity.

Comments from the JPMA member companies on Alternative Testing

Fifty-two companies responded. Most of the comments were divided between acceptance and rejection of alternative testing for animal studies. The opinions were sometimes mixed, even in the same company, which made a clear understanding of opinion difficult. But most of the time, progressive and conservative opinions were split evenly down the center. The opinion of the progressive group was that alternative testing would be useful in screening and mechanistic analyses of toxicity. Those of the conservative opinion pointed out that the new methods had not yet been organized enough by the regulatory authorities, that they had not been established or validated as alternatives to current toxicology studies, and that there was insufficient correlation between *in vivo* and *in vitro* study results.

Animal welfare was mentioned by both groups. The conservative group thought the problem could be handled by reducing the number of animals used in current toxicology studies. Other comments indicated that alternative

testing methods should be developed for extrapolation of results to humans rather than to laboratory animals, and that relevant information about the testing should be available so that alternative testing methods could be used more frequently.

Discussion

The survey results indicated that about 60% of the companies had done some alternative testing one way or another. When correlation of alternative testing results to study results in humans, study costs, and animal welfare issues were considered by the pharmaceutical industry, it was recognized that the subject had been actively discussed. The main purposes of the tests were mechanistic analyses of toxicity and screening, but some of the test methods had not yet been established, and it was expected to take some time before they were validated. Cooperation and discussion by many companies are needed to establish alternative test methods.

Many companies recognized the future need for alternative testing for screening and mechanistic analyses of toxicity, but only a few companies have established these test systems and have done the tests routinely. At present, alternative testing is not better than *in vivo* toxicity testing; however, when alternative testing methods are established and serve to clarify mechanisms of toxicities, they could aid in the extrapolation of results to humans. Much research is necessary to establish these alternative methods.

About half the companies are doing some research and development as an aid in establishing alternative testing. It is very important to pursue this research in consideration of future needs. Further developments in the already advanced fields of hepatic toxicity, renal toxicity, teratogenicity, and local irritation are expected due to social trends (animal welfare and environmental issues).

The present purposes for testing are mechanistic analyses of toxicities and screening, but of equal importance is establishing an accurate correlation with *in vivo* studies and convince the regulatory authorities to accept data from alternative testing as submittal information for new drugs.

There is a fourth R concerning the alternative testing concept. Gad (1990) reviewed the previous first three R's of replacement, reduction and refinement. A fourth R, responsibility, was not in the first proposal. Research toxicologists must stand by their responsibility to be conservative in ensuring safety. At the same time, the responsibility of regulatory toxicologists and regulatory agents must be recognized to accept IND and NDA. Also, the correlation between *in vivo* toxicity and *in vitro* toxicity must be monitored closely and constantly.

During the past decade, issues of animals use and care in toxicological research and testing have become one of the fundamental concerns of both scientists and the general public.

To evaluate alternative testing in mammalian toxicity studies, regulatory toxicologists and regulatory agents should provide a current overview of the general concepts, status and progress of *in vitro* alternatives to research toxicology. The general public clearly supports animal use in research when the need and benefit are perceived.

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