Novel cytotoxicity test based on menadione-catalyzed H₂O₂ productivity for food safety evaluation

Shiro Yamashoji¹ & Kenji Isshiki²

¹ Kobe Gakuin Women's College, 27-1 Hayashiyama-cho, Nagata-ku, Kobe 653-0861, Japan

² National Food Research Institute, The Ministry of Agriculture, Forestry and Fisheries, 2-1-2 Kannondai, Tsukuba 305-8642, Japan

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Abstract

Menadione-catalyzed H₂O₂ production by viable cells was proportional to viable cell number, and the assay of this H₂O₂ production was applied to the cytotoxicity test of 17 substances which were used for international validation of fixed-dose procedure as an alternative to the classical LD₅₀ test. The cytotoxicity of substances tested was observed 4 h after the incubation with animal cells, and the viability was determined in 10 min according to menadione-catalyzed H₂O₂ production assay. IC₅₀ of each substance required for 50% inhibition of menadione-catalyzed H₂O₂ production was similar among HepG2, HuH-6KK, HUVE, Vero, Intestine407, NIH/3T3 and Neuro-2a cells. Twelve substances, 3 substances and 2 substances showed the difference of one, two and three orders in the magnitude between LD₅₀ and IC₅₀, respectively. These results show that menadione-catalyzed H₂O₂ production assay is useful for the rapid detection of toxic compounds having the basal cytotoxicity common to various cells, but is unfit for the detection of organ-specific toxic compounds.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium; NR, neutral red; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide; DA-64, N-(carboxymethylaminocarbonyl)-4-4'-bis(dimethylamino)-diphenylamine sodium salt; Bis-MAPS-C3, Bis[4-(N-propyl-N-sulfopropyl)amino-2,6-dimethyl-phenyl]methane disodium salt; FGF, Fibroblast growth factor; LD₅₀, 50% lethal dose; IC₅₀, 50% inhibition concentration for menadione-catalyzed H₂O₂ production; HepG2, Human hepatic tumor cells HuH-6KK, Human hepatoblastoma cells; Vero, African green monkey kidney cells; Intestine407, Human fetus intestine cells; NIH/3T3, Swiss mouse embryo cells; HUVE, Human umbilical vein endothelial cells; Neuro-2a, Mouse neuroblastoma cells

Introduction

The general accepted methods for assessing acute oral toxicity are based on graduated-dose procedures in which groups of laboratory animals are exposed to a range of dose levels with the top dose chosen to produce mortality in more than half of the group. These procedures were described in an internationally agreed guideline published by the Organization for Economic Cooperation and Development (OECD, 1981). In 1984, a new approach to acute toxicity testing was suggested by the British Toxicity Society (BTS)

based on a fixed-dose procedure (BTS, 1984). This avoided using death of animals as an endpoint, and relied instead on the observation of clear signs of toxicity developed at one of a series of fixed dose levels. Furthermore, fixed-dose procedure was expected to contribute to a reduction in the number of animals tested (Van den Heuvel et al., 1990; Whitehead and Curnow, 1992; Yam et al., 1991).

The international validation of a fixed-dose procedure as an alternative to the classical LD₅₀ test was performed by 33 laboratories in 11 countries (Van den Heuvel et al., 1990). The acute toxicity

to the rat of 20 substances was evaluated. The test substances were distributed in November 1988 and all tests were completed by July 1989. The study has shown that the fixed-dose approach to acute oral toxicity testing: (1) produces consistent results that are not substantially affected by inter-laboratory variations; (2) provides adequate information for risk assessment purposes on signs of toxicity; (3) uses fewer animals than the current internationally agreed OECD procedure (Guideline 401-revised); (4) subjects animals to less pain and distress than the classical LD₅₀ test and causes less compounds-related mortality; and (5) enables substances and preparations to be ranked according to the classification system by Commission of the European Communities on the basis of their acute oral toxicity.

On the other hand, another approach as an alternative to the above animal tests has been proposed in order to promote the screening of toxic compounds and clarify the mechanism of toxicity in mammalian cell cultures (Cook and Mitchell, 1989). These cytotoxicity tests are rapid and cheap for the determination of dose-response compared with animal tests, but it will be difficult to replace animal tests because of organ-specific toxicity and characteristic pharmacokinetics of many substances (Ekwall, 1983). Thus, cytotoxicity tests are expected to be useful for the detection of a basal cytotoxicity common to various cells, tissues and organs.

The present study makes clear the difference in toxicity level between the cytotoxicity test and animal tests in order to propose a novel rapid cytotoxicity test. The viability of cells exposed to toxic compounds was rapidly determined by the assay of menadione-catalyzed H₂O₂ production by viable cells which keep both the activity of plasma membrane NAD(P)H: quinone oxidoreductase and the production of NAD(P)H as shown in Figure 1 (Yamashoji et al., 1991; Yamashoji, 1998). The viable cell number is proportional to H₂O₂ concentration 10 min after the addition of menadione to cell culture medium (Yamashoji et al., 1992).

The results obtained show that most of the test substances used for the above international validation of fixed-dose procedure have similar cytotoxicity level among cells prepared from various organs, but have different toxicity level between cytotoxicity test (IC₅₀) and animal tests (LD₅₀). Thus, this cytotoxicity test is expected to be useful for the rapid detection of toxic compounds inhibiting general cell functions.

The conditions for the detection of cytotoxicity by this cytotoxicity test are also discussed in this study.

Materials and methods

Organism and growth conditions

HepG2, HuH-6KK, Vero, Intestine 407 and NIH/3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum. HUVE cells were cultured in MCDB131 supplemented with 10% fetal bovine serum and FGF (10 ng/ml). Neuro-2a cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum. The cells were grown on tissue dishes in a humidified atmosphere of 95% air and 5% CO₂ at 37 °. Exponentially growing cells were trypsinized, and single cells obtained were collected by centrifugation and were resuspended in a 96-well tissue culture plate. The cell number was 40 000 cells/well, and the cells were cultured overnight under the above conditions. The origins of cell line, media and culture plate were as follows: HepG2, Vero and NIH/3T3 from Dainippon Pharmaceutical Co., Ltd.; HUVE and FGF from Morinaga Seika Co., Ltd.; HuH-6KK from National Food Research Institute; fetal bovine serum from Life Technologies Oriental, Inc.; DMEM and RPMI1640 from Sigma Chemical Co.; culture plate from Falcon Labware.

Cytotoxicity test

To 100 µl of cell culture medium in each well of 96-well tissue culture plate 5 µl of test solution was added, and the mixture was incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37 ° for 4 h. Each well was washed twice with 100 µl of MEM, and 100 µl of MEM containing 0.2 mM menadione was added to each well. After 10 min-incubation in a humidified atmosphere of 95% air and 5% CO₂, 150 µl of the reagent solution was added to each well, and the absorbance at 725 nm in each well was measured with a microplate reader 1 min later. The reagent solution contained 0.1% bovine albumin, 10 U/ml peroxidase, 200 µM DA-64 in 1 M acetate buffer (pH 5.0). The viability of the cells exposed to substances was determined as a percentage based on the absorbance of well containing intact cells. DA-64 was purchased from Wako Pure Chemical Industries Ltd.

Figure 1. Proposed mechanism for menadione-catalyzed H_2O_2 production by viable cells.

Table 1. Menadione-catalyzed H_2O_2 production by various cells

Cell lines	H_2O_2 concentration ($\mu\text{M}/10 \text{ min}$)
HepG2	14.3
HuH-6KK	11.5
HUVE	8.2
Intestine 407	4.4
Neuro-2a	12.1
NIH/3T3	22.5
Vero	5.0

Each values is the mean of four determinations. The standard derivations were $< \pm 8\%$.

Results

Menadione-catalyzed H_2O_2 production

Table 1 shows the menadione-catalyzed H_2O_2 production by various cells reaching to about 80% confluent in a 96-well tissue culture plate under the experimental conditions. NIH/3T3 cells had the highest activity, and Intestine407 had the lowest activity among the cells tested. NIH/3T3 cells showed good growth rate and uniform cell layer, but HepG2, HuH-6KK and Neuro-2a cells formed colony. As menadione-catalyzed H_2O_2 production is carried out on the surface of cells, the H_2O_2 production by uniform cell layer is expected to get predominance over that by

colony. Furthermore, menadione-catalyzed H_2O_2 production depends on both NAD(P)H production and NAD(P)H: quinone oxidoreductase activity in each cell.

Selection of solvent and medium

Table 2 shows the cytotoxic effect of solvent on various cells. Acetonitrile and 70% ethanol had lower cytotoxic effects than dimethylformamide which were harmful for HUVE, NIH/3T3, Vero and Neuro-2a cells. When hydrophobic compounds were completely solubilized in dimethylsulfoxide, turbidity or the thin layer on medium was observed upon the addition of the mixture into medium because of poor solubility of hydrophobic compounds in medium. For example, ferrocene and naphthalene caused turbidity and thin layer, respectively. These problem will cause the poor contact of the compounds to cells and the difficulty to evaluate their cytotoxicity. In order to evaluate the effect of solvent on the cytotoxic effects of substances, dimethyl sulfoxide and 70% ethanol were tested with HepG2 and HuH-6KK cells. Ferrocene and naphthalene dissolved in dimethyl sulfoxide had same or greater cytotoxic effect on HuH-6KK and HepG2 cells than those dissolved in 70% ethanol, respectively (Table 3). On the other hand, phenyl mercury acetate and 4-aminophenol had greater cytotoxic effect on both HepG2 and HuH-6KK cells than those dissolved in dimethylsulfoxide (Table 3). These facts suggest

Table 2. Cytotoxic effect of solvent on menadione-catalyzed H₂O₂ production by various cells

Solvent	Viability (%)						
	HepG2	HuH-6KK	HUVE	Intestine407	Neuro-2a	NIH/3T3	Vero
Acetonitrile	90	96	84	97	100	79	92
Dimethylformamide	57	46	0	73	29	21	24
Dimethylsulfoxide	63	80	10	105	51	78	34
70% Ethanol	105	81	63	108	79	90	100

The final concentrations of ethanol and other solvent in culture medium were 3.5 and 5% (V/V), respectively. Viability is the mean of four determinations. The standard deviation were $< \pm 10\%$.

Table 3. Effect of solvent on cytotoxic effect of substances

Substances		IC ₅₀ (%)			
		HepG2		HuH-6KK	
		DMSO	70% Ethanol	DMSO	70% Ethanol
A.	Nicotine	0.15	0.19	0.38	0.32
B.	Sodium pentachlorophenate	0.006	0.011	0.024	0.024
C.	Ferrocene	0.004	0.004	0.005	0.086
D.	2-Chloroethylalcohol	1.1	1.1	1.1	2.4
E.	Sodium arsenite	1.1	1.9	1.1	0.4
F.	Phenyl mercuryacetate	0.0059	0.0009	0.0062	0.0003
G.	p-Dichlorobenzene	0.007	0.060	0.014	0.058
J.	Acetanilide	0.042	0.36	0.75	0.35
K.	Quercetin 2H ₂ O	0.015	0.005	0.009	0.022
M.	Piperidine	0.04	0.07	0.07	0.08
N.	Mercuric chloride	0.0050	0.0045	0.0020	0.0020
P.	1-Phenyl-2-thiourea	0.25	0.34	0.52	0.34
R.	4-Aminophenol	0.031	0.0018	0.033	0.0006
T.	Naphthalene	0.004	0.052	0.021	0.061
U.	Acetonitrile	>5	>5	>5	>5
X.	Resorcinol	0.95	0.75	1.00	0.34
Y.	Dimethylformamide	11.1	10.5	12.0	7.0

Substances (A–Y) were dissolved in dimethylsulfoxide (DMSO) or 70% ethanol, and added to cell culture. The final concentrations of ethanol and DMSO were 3.5 and 5% (V/V), respectively. IC₅₀ is the percentage of weight per volume (g/ml) and is the mean of four determinations. Standard derivations were $< \pm 10\%$.

that the initial conditions for solubility of compounds affect the sensitivity of cells to toxic compounds. From the above results, 70% ethanol was expected to be desirable for the evaluation of cytotoxic compounds by menadione-catalyzed H₂O₂ production assay because of its low cytotoxicity and strong sterilization effect.

The effects of fetal bovine serum on the cytotoxic effects of substances was examined because their cytotoxic effects were expected to depend on the absorption by serum proteins and/or the interaction with other components. Table 4 shows little effects of the serum on the cytotoxic effects of substances. On the basis of this fact, the direct addition of the test solution

to medium containing 10% fetal bovine serum was performed for this cytotoxicity test.

Cytotoxicity test with various cells

Seventeen substances were dissolved in 70% ethanol and were added to various cell culture. Table 5 shows the cytotoxic effects of 17 substances on cells after 4 h-incubation. All cells tested showed the same tendency of sensitivity to 17 substances (Table 5). None of cells tested showed the sensitivity to acetonitrile. NIH/3T3 cells had the highest sensitivity to 8 substances, but the lowest sensitivity to ferrocene, quercetin and 1-

Table 4. Effect of serum on cytotoxic effects of substances

Substances	IC ₅₀ (%)	
	+10% serum	– serum
A	0.15	0.23
B	0.006	0.011
C	0.004	0.004
D	1.1	0.8
E	1.1	1.0
F	0.0059	0.0050
G	0.007	0.003
J	0.042	0.57
K	0.015	0.044
M	0.04	0.03
N	0.0050	0.0047
P	0.25	0.48
R	0.031	0.025
T	0.004	0.003
U	>5	>5
X	0.95	0.84
Y	11.1	10.4

Each letter (A–Y) represents the substances in Table 3. Substances (A–Y) were dissolved in dimethylsulfoxide and added to cell culture. Final concentration of dimethylsulfoxide in culture medium was 5% (V/V). HepG2 cells were used for this test. IC₅₀ is the percentage of weight per volume (g/ml) and is the mean of four determinations. Standard derivations were $< \pm 10\%$.

phenyl-2-thiourea among cells. HepG2, HuH-6KK and HUVE cells showed the sensitivity to cytotoxic effects of 16 substances. Though HUVE cells had the same sensitivity to 16 substances as HepG2 cells, it was difficult for the cells to show clear cell viability because of low menadione-catalyzed H₂O₂ production activity. Vero and Intestine407 cells were also not useful for the cytotoxicity test because of low activity of menadione-catalyzed H₂O₂ production. Neuro-2a cells had high activity of menadione-catalyzed H₂O₂ production, but the cells had the lowest sensitivity to 1-phenyl-2-thiourea and naphthalene. From the above results, HepG2 and HuH-6KK cells would be the most useful among the cells tested for the detection of cytotoxic effects of 16 substances other than acetonitrile.

Comparison of LD₅₀ and IC₅₀

As shown in Table 6, LD₅₀ and IC₅₀ were divided into 3 and 5 groups every one order, respectively. In the

case of 12 test substances (B,C,F,G,J,M,N,P,T,U,X and Y), the magnitude of the difference between LD₅₀ and IC₅₀ was one order. A,E and K showed the difference of two order, and D and R showed the difference of three orders between LD₅₀ and IC₅₀. When LD₅₀ is much lower than IC₅₀, the substances are expected to have organ-specific toxicity. For example, nicotine has been considered to interfere with neurotransmission specifically. In the opposite case, the cytotoxic substances may not be absorbed into organ or may be converted to non-toxic compounds by organs. The substances having small difference between LD₅₀ and IC₅₀ may have basal cytotoxicity common to various cells, tissues and organ. Though it is not reasonable to directly compare LD₅₀ with IC₅₀, the comparison of LD₅₀ and IC₅₀ may contribute to the distinctions between organ-specific toxins and non-specific toxins.

Discussion

Menadione-catalyzed H₂O₂ production assay (Yamashoji et al., 1992) is very rapid and sensitive to cytotoxic events compared with conventional MTT reduction assay (Mosman, 1987) and neutral red inclusion assay (Borenfreund and Puerner, 1989). The mechanism of extracellular menadione-catalyzed H₂O₂ production by intact cells has been proposed by study using yeast cells and mammalian cells. Recently, menadione-catalyzed H₂O₂ production by mammalian cells as well as yeast cells was found to be catalyzed by plasma membrane-bound NAD(P)H:quinone oxidoreductase (Yamashoji, 1998). At first the assay of menadione-catalyzed H₂O₂ production was performed by chemiluminescent assay, and then was modified with high sensitive colorimetric reagent to H₂O₂ in order to establish the conventional colorimetric assay. DA-64 was useful for the determination of H₂O₂ concentration range from 0.5 to 20 μ M. Recently, Bis-MAPS-C3 produced as a new chromogenic hydrogen donor was also confirmed to be excellent for the assay of menadione-catalyzed H₂O₂ production range from 1 to 20 μ M (Mizoguchi, 1993). As menadione interferes with the hydrogen transfer from chromogenic hydrogen donor into H₂O₂ in peroxidase-catalyzed oxidation reaction or converts the resulting pigment to the colorless compound by oxidation of the pigment, the attention should be paid for the selection of the chromogenic hydrogen donor for menadione-catalyzed H₂O₂ production assay.

Table 5. Cytotoxicity of substances on menadione-catalyzed H₂O₂ production by various cells

Substances	IC ₅₀ (%)							
	HepG2	HepG2*	HuH-6KK	HUVE	Intestine407	Neuro-2a	NIH/3T3	Vero
A	0.19	0.63	0.32	0.19	0.15	0.32	0.15	0.15
B	0.011	0.013	0.024	0.013	0.013	0.004	0.042	0.044
C	0.040	0.050	0.086	0.025	0.019	0.088	>0.1	0.082
D	1.1	1.1	2.4	2.3	5.2	1.1	4.7	2.6
E	1.9	1.1	0.4	0.2	>2.2	0.8	0.1	>2.2
F	0.0009	0.0002	0.0003	0.0005	0.0005	0.0006	0.0005	0.0009
G	0.060	0.027	0.058	0.057	0.021	0.056	0.028	0.034
J	0.36	0.16	0.35	0.32	0.35	0.18	0.05	0.39
K	0.005	0.020	0.022	0.007	0.004	0.003	>0.07	0.068
M	0.07	0.04	0.08	0.14	0.015	0.08	0.03	0.04
N	0.0045	0.0010	0.0020	0.0006	0.0022	0.0010	0.0104	0.0024
P	0.34	0.19	0.34	0.44	>0.52	>0.52	>0.52	0.39
R	0.0018	0.0007	0.0006	0.0003	0.0017	0.0003	0.0003	0.0013
T	0.052	0.017	0.061	0.039	0.107	>0.11	0.017	0.035
U	>5	>5	>5	>5	>5	>5	>5	>5
X	0.75	0.19	0.34	0.33	0.41	0.16	0.08	0.33
Y	10.5	9.1	7.0	3.5	9.8	3.5	3.0	3.4

Each letter (A–Y) represents the substances in Table 3. Substances (A–Y) were dissolved in 70% ethanol and added to cell culture. Final concentration of ethanol was 3.5% (V/V). IC₅₀ is the percentage of weight per volume (g/ml) and is the mean of four determinations. The standard derivations were $< \pm 10\%$.

* Tripsinized HepG2 cells were mixed with substances. The cell number was 50 000 cells/well.

Table 6. Comparison of LD₅₀ and IC₅₀ of substances

Range of dose (%)	LD ₅₀ (%)	IC ₅₀ (%)
0.001 ~ 0.0001		F, R
0.01 ~ 0.001	A, D, E, F	K, N
0.1 ~ 0.01	B, M, N, P, X	B, C, G, M, T
1 ~ 0.1	C, G, J, K, R, T, U, Y	A, E, J, P, X
>1		D, U, Y

Each letter (A–Y) represents the substances in Table 3. Percent in LD₅₀ means % in weight per body weight on the basis of data of Van den Heuvel et al. (1990). IC₅₀ means the average of percentage of weight per volume (g/ml) in culture medium on the basis of data shown in Table 5.

The difference in the magnitude between minimum and maximum IC₅₀ was below one order in the case of substances other than C, E, K, P, T and U which have little cytotoxicity to some cell lines at the concentration of the maximum solubility in the assay system. In the case of C, P and T, the difference in the magnitude between minimum and maximum IC₅₀ was one order when IC₅₀ was determined under the conditions that these substances were completely solubilized in the assay system. Thus, 13 substances among 17 substances had the similar cytotoxic effects on menadione-catalyzed H₂O₂ production by various

cell lines because substance U had little cytotoxic effect in this experiment. These facts suggest that most of substances tested have basal cytotoxicity common to various cell lines.

In this study IC₅₀ of substances tested was not proportional to LD₅₀. IC₅₀ of 12 substances among 17 substances was less or more than LD₅₀ at the range of one order of magnitude. These substances are expected to have toxicity common to general cell functions in the animal. LD₅₀ of substances A, D and E was less than IC₅₀ at the range of two or three orders, suggesting that they have toxicity to organ-specific functions. Thus, these organ-specific toxins cannot be studied in cultures of less differentiated cells. On the other hand, substances K and R showed much less IC₅₀ compared with LD₅₀. Substances K and R may injure the cell culture with a very low cell density rather than animals with multi-compartment and high cell density system. These results and speculation show that it will be difficult to replace animal testing by cell tests in toxicity evaluation because of the organ-specific toxicity and characteristic pharmacokinetics of many substances as proposed by Ekwall.

As the cytotoxicity takes advantage in the detection of basal cytotoxins common to less differentiated

cells, it will be expected that the cytotoxicity test with more differentiated cells contributes to the detection of organ-specific toxins. For example, the cells with the specific receptors of neurotransmission will be sensitive to the drugs interfering with neurotransmission. The approach of cytotoxicity test to animal test will depend on the development of more differentiated cell culture system with organ-specific functions. *In vitro* cytotoxicity test, such as our trial, would be very useful to obtain the basal data for food safety and other safety assessments. These trial could contribute to reduce the number of experimental animals sacrificed.

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