VITOTOX™ TEST FOR GENOTOXIC SCREENING OF PHARMARCEUTICALS

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ABSTRACT

The Vitotox™ test is a high throughput bacterial genotoxicity assay which can detect DNA damage caused by genotoxic compounds as light emission changes depending on SOS DNA repair induction.

INTRODUCTION

The Vitotox™ test is a high throughput bacterial genotoxicity assay, which can detect DNA damage caused by genotoxic compounds. This test utilizes the bacterial SOS DNA-repair system induced by genotoxic compounds [2, 3]. Two genetically engineered *Salmonella typhimurium* strains are used in this test system, TA104 *recN*2-4 (Genox strain) and TA104 *pr1* (Cytox strain). The former strain carries a plasmid containing the bacterial luciferase operon (*luxCDABE*) of luminous bacteria *Vibrio fischeri* under transcriptional control of an *recN* promoter (*recN*2-4). The latter strain constitutively expresses the *lux* operon. Genotoxic compounds activate the *recN* promoter in the Genox strain, which results in transcriptional induction of the *lux* operon followed by the enhancement of light emission. The cytotoxicity of the compounds is simultaneously assayed with reference to the Cytox strain to identify the non-specific enhancement of light emission. Concomitant use of the Genox and the Cytox strains allows us to identify false positive results caused by non-specific light emission induced by other mechanisms, and not by the genotoxic effect.

MATERIALS AND METHODS

Materials

compounds were purchased from GENAUR Molecular Products.

Two tester strains, *S. typhimurium* TA104 *rec*N2-4 (Genox strain) and TA104 *pr1* (Cytox strain) were supplied as components of the Vitotox 10 Kit obtained from GENTAUR Insrtitute (Kampenhout, Begium). Rat liver S9, purchased from Kikkoman Co. (Chiba, Japan), was prepared as a 9000x g supernatant fraction of the liver homogenates prepared from male SD rats treated with phenobarbital and 5,6-benzoflavone. Co-factor I was purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). The S9 mix was prepared by mixing the S9 fraction and co-factor solutions at the volume ratio of 1:9.

Kit Contents

1. 5 Yellow vials of 0.1ml Genox reagent *S. typhimurium* TA104 *rec*N2-4 2. 5 Blue vials of 0.1 ml Cytox reagent *S. typhimurium* TA104 *pr1* (Cytox strain) store at -85°C

3. 10 vials 8 ml Rich Growth Solution (with enhancer) -20°C

4. 5 vials of 10 ml Poor Growth Solution -20°C

5. White 96 well plate 5 pcs

6. Clear 96 well strip plates 3pc

7. Reagent basin 20

Equipment

Luminometer

Orbital Shaker with temperature control 35 - 37°C

Photometer 590 nm

12 channel pipette 5 -50 ul

12 channel pipette 50-300 ul

Single channel pipettes 10-100ul, 20-200ul, 1-5ml

Assay procedure

Cultures:

Day 1

1. Remove 2 vials of 8ml Rich Growth Solution and 1 vials of Genox and Cytox reagent

from the freezer and leave until room temp RT

2. Add 0.1ml of each to their destinated 8 ml Rich Growth Medium

3.Use the Medium Vial as culture vessel and incubate the vials overnight (18h) on an orbital shaker (160-180 RPM, corresponding to 0.27-0.35G)at 35 to 37°C

Day 2

Remove 10 ml of Poor Growth Medium from the freezer and leave until room temperature RT

Measure the optical density at 590nm with 0.5ml that you remove from the 8ml

The optical densitys are

Genox reagent 0.200-0.500

Cytox reagent 0.400-0.600

Store the vials that are in the correct range and store at 4°C until use.

If the densities are below, the growth time can be extended up to 20h.

Dilute each bacterial suspension as described:

Genox reagent: Add 1.1 ml of the 7.5 ml bacterial suspension and add to the 10 ml of the Poor Growth Medium (1/10 dilution). Discard the rest of the Genox reagent (7.9 ml)

Cytox reagent: Discard 2.5ml of the 7.5 ml bacterial suspension and mix the remaining 5 ml to 5 ml sterile distilled water. (1/2 dilution)

You will have 11.1ml Genox culture and 10 ml Cytox culture ready for use. You will only need +- 4.08 ml from each. (2x 1080ul and 2x 960ul)

Dilution of samples

A dilution series of the sample is used for the test. Dilutions of one sample are most easily done into two 12 well microstrips included in the kit. Testing of one sample according to this procedure uses four rows (A-D) of the 96 well plate. It is recommended to test another sample simultaneously using the four remaining rows of wells E-H. he dilution of the samples is done

The Vitotox-384 test

The Vitotox-384 test was performed according to the instructions for the Vitotox 10 Kit supplied by the manufacturer, with some minor modifications.

The lyophilized test bacteria (Genox and Cytox strains) in the vials were hydrated with the growth medium and incubated over night at 37°C with shaking. Overnight cultures of these bacterial strains were diluted with the medium to make an optical density 0.03.

Each test substance was dissolved or suspended in DMSO and a series of test substance solutions was prepared by 2-fold dilutions with the same solvent. Each preparation was diluted ten-fold with purified water, and the resultant mixtures were applied to the treatment. For our compounds, 1000 μg/mL was selected as the highest concentration. When it was impossible or technically difficult to transfer the test substance preparations to the treatment plate (e.g., inability of pipetting due to large precipitates), a lower concentration was selected as the highest concentration. The solvent was used as negative control. 4-Nitroquinoline (4-NQO) and benzp[a]pyrene (B[a]P) were used as the positive control in the treatment with and without metabolic activation, respectively.

Five μL of the test preparations was added to each well of a 384-well microplate. Five μL of the S9 mix or purified water and 40μL of the bacterial cultures were added to each well. The light production from each well was measured every 15 min for 4 hr at 30°C using a luminometer (Fluoroskan Ascent FL, ThermoLabsystems).

The data was analyzed with Ascent Software that automatically calculated the Genox/Cytox ratio. The genotoxicity of the test substance was evaluated with the Genox/Cytox ratio. When the ratio increased dose-dependently and became 1.5 or higher of the solvent control value in non-cytotoxic concentrations, the test substance was judged positive for genotoxicity (DNA-damaging activity). When the test substance

showed a severe cytotoxicity, an additional test was conducted at the lower concentrations.

The Ames test

The Ames test was performed with the *Salmonella typhimurium* TA98, TA100 and TA2637, and *Escherichia coli* WP2uvrA, both in the presence and absence of metabolic activation with rat S9 mix [4].

Table 1 Results of the Vitotox-384 test and comparison with other genotoxicity tests

Test compounds	CAS No.	Source[a)]	Vitotox-384 (present study)			Vitotox-96		Vitotox	Ames	SOS	*umu*	Carcinogenicity
			-S9	+S9	Highest dose (nM)	-S9	+S9					
Ames-positive compounds												
9-Aminoacridine (9-AA)	134-50-9	S	-	-	7.8	I	I	N.A.	+	+/-	(+)	N.A.
2-Aminoanthracene (2-AA)	613-13-8	W	-	+	3.1 x 10	-	+	N.A.	+	+	+	+
2-Aminofluorene (2-AF)	153-78-6	W	-	+	3.5×10^2	-	+	+	+	+	N.A.	+
2-Amino-3-methyl-3H-imidazo[4,5-*f*]quinoline (IQ)	76180-96-6	W	-	+	6.1	-	+	N.A.	+	N.A.	+	+
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (P	105650-23-5	W	-	+	1.8×10^2	-	+	N.A.	+	N.A.	+	+
3-Amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2)	72254-58-1	W	-	+	2.3 x 10	-	+	N.A.	+	N.A.	+	+
5-Azacytidine	320-67-2	W	+	+	2.5	+	+	N.A.	+	N.A.	N.A.	+
Azobenzene	103-33-3	W	-	+	2.7×10^3	-	+	N.A.	+	N.A.	(+)	+
Benzo[*a*]pyrene (B[a]P)	50-32-8	T	-	+	2.5×10^2	-	+	+	+	+	+	+
Daunorubicin (Dau)	23541-50-6	W	+	+	1.7 x 10	+	+	N.A.	+	+	+	+
2,4-Diaminotoluene (2,4-DAT)	95-80-7	W	I	I	8.2×10^5	I	I	N.A.	+	N.A.	(+)	+
2,6-Diaminotoluene (2,6-DAT)	823-40-5	T	I	I	8.2×10^5	I	I	N.A.	+	N.A.	(+)	-
1,3-Dichloropropene	542-75-6	W	+	+	1.1×10^5	+	+	N.A.	+	+	N.A.	+
Diethylnitrosamine (DEN)	55-18-5	W	-	+	9.8×10^5	-	+	+	+	+	(+)	+
7,12-Dimethylbenzanthracene (DMBA)	57-97-6	W	-	+	1.4×10^2	-	+	N.A.	+	+	+	+
1,2-Dimethylhydrazine (DMH)	57-14-7	T	I	I	7.5×10^5	I	I	N.A.	+	N.A.	+/-	+
Dimethylnitrosamine (DMN)	62-75-9	W	-	+	1.3×10^5	-	+	N.A.	+	+	+	+
1,2-Epoxybutane	106-88-7	W	-	-	1.1×10^5	-	-	N.A.	+	+	+	+
Ethidium bromide (EtBr)	1239-45-8	S	-	+	3.8×10^2	-	+	N.A.	+	+/-	+	+
Ethyl methanesulfonate (EMS)	62-50-0	N	+	+	8.1×10^4	+	+	+	+	+	+	+
N-Ethyl-*N*'-nitro-*N*-nitrosoguanidine (ENNG)	4245-77-6	T	+	+	4.9 x 10	+	+	N.A.	+	+	+	+
Furylfuramide (AF-2)	3688-53-7	W	+	+	3.2×10^{-1}	+	+	N.A.	+	+	+	+
8-Hydroxyquinoline	148-24-3	T	-	-	6.9×10^2	-	-	N.A.	+	-	-	-
Hydroxyurea	127-07-1	S	+	+	1.3×10^5	I	I	N.A.	+	+	+	I
ICR191	17070-45-0	W	+	+	2.1×10^5	+	+	+	+	+	N.A.	N.A.
3-Methylcholanthrene (3-MC)	56-49-5	S	-	+	1.1×10^2	-	+	N.A.	+	+	+	+
Methyl methanesulfonate (MMS)	66-27-3	T	+	+	5.8×10^3	+	+	+	+	+	+	+
N-Methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG)	70-25-7	S	+	+	1.6×10^2	+	+	N.A.	+	+	+	+
Mitomycin C (MMC)	50-07-0	W	+	+	7.5	+	+	+	+	N.A.	+	+
2-Nitrofluorene (2-NF)	607-57-8	S	+	+	6.0×10^2	+	+	N.A.	+	+	+	+

Table 1 (continued)

Test compounds	CAS No.	Source[a)]	Vitotox-384 (present study)			Vitotox-96		Vitotox	Ames	SOS	*umu*	Carcinogenicity
			-S9	+S9	Highest dose (nM)	-S9	+S9					
4-Nitroquinoline-*N*-oxide (4NQO)	56-57-5	W	+	+	2.6×10^3	+	+	+	+	+	+	+
N-Nitrosomethylurea (NMU)	684-93-5	S	+	+	4.9×10^3	+	+	N.A.	+	+	+	+
Styrene oxide	35311	W	-	-	2.6 x 10	-	-	N.A.	+	+	+	+
Ames-negative compounds												
Actinomycin D	50-76-0	S	-	-	8.0×10^3	-	-	N.A.	-	-	-	+
Allyl isovalerate	2835-39-4	W	-	-		N.A.	N.A.			N.A.	-	+
Benzoin	119-53-9	W	-	-	4.7×10^4	-	-	N.A.	-	N.A.	-	-
Bisphenol A	80-05-7	T	-	-	4.4×10^4	-	-	N.A.	-	N.A.	N.A.	+/-
Butylated hydroxyanisol	25013-16-5	W	-	-	2.8×10^3	-	-	N.A.	-	-	-	-
Caffein	58-08-2	S	-	-	5.1×10^4	-	-	N.A.	-	-	-	-
Caprolactam	105-60-2	W	I	I	8.8×10^4	I	I	N.A.	-	N.A.	-	-
Chlorodibromomethane	124-48-1	W	-	-	4.8×10^4	-	-	N.A.	-	N.A.	+	-
3-Chloro-2-methylpropene	563-47-3	W	-	-	1.1×10^4	-	-	N.A.	-	N.A.	+	+
Chlorpheniramine maleate	113-92-8	S	-	-	2.6×10^4	-	-	N.A.	-	N.A.	N.A.	+
Chromotrope FB	3567-69-9	S	-	-	2.0×10^4	-	-	N.A.	-	N.A.	N.A.	+
DDT	50-29-3	T	-	-	8.8×10^4	-	-	N.A.	-	-	N.A.	+
Di(2-ethylhexyl)phthalate	117-81-7	W	-	-	2.6×10^4	-	-	N.A.	-	N.A.	N.A.	+
Diethylstilbesterol	56-53-1	T	-	-	3.7×10^4	-	-	N.A.	-	N.A.	-	+
5-Fluorouracil	51-21-8	S	-	-	1.2×10^3	-	-	N.A.	-	+/-	+	-
Geranyl acetate	105-87-3	W	-	-		N.A.	N.A.	N.A.	-	-	-	-
Isophorone	78-59-1	T	I	I	7.2×10^4	I	I	N.A.	-	N.A.	+	+
d-Mannitol	69-65-8	W	-	-	5.5×10^4	I	I	N.A.	-	N.A.	-	-
dl-Menthol	15356-70-4	T	-	-	6.4×10^4	-	-	N.A.	-	-	N.A.	-
Methoxychlor	72-43-5	S	-	-	2.9×10^4	-	-	N.A.	-	N.A.	-	-
Phenobarbital	50-06-6	W	-	-	4.3×10^4	-	-	N.A.	-	N.A.	-	+
Reserpine	50-55-5	W	-	-	1.6×10^4	-	-	N.A.	-	N.A.	+/-	+
Saccharin sodium	128-44-9	W	-	-	4.9×10^4	-	-	N.A.	-	N.A.	-	+
Safrole	94-59-7	T	-	-	6.2×10^4	-	-	N.A.	-	-	-	+
Sulfisoxazole	127-69-5	S	-	-	3.7×10^4	-	-	N.A.	-	N.A.	N.A.	-
Thioacetamide	62-55-5	W	-	-	1.3×10^4	I	I	N.A.	-	N.A.	-	+
Urethane	51-79-6	S	I	I	1.1×10^4	I	I	N.A.	-	-	-	+
WY-14643	50892-23-4	S	-	-	3.1×10^4	-	-	N.A.	-	N.A.	N.A.	+

+ = positive; - = negative; +/- = equivocal; (+) = positive in the particular condition; I = inconclusive; N.A.= not available; a) These componds were obtained from the following sources; N, Nacalai Tesque Inc. (Kyoto, Japan); S, Sigma-Aldrich (St. Louis, U.S.A.); T, Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan); W, Wako Pure Chemical Industries Ltd. (Osaka, Japan).

RESULTS AND DISCUSSION

Table 1 summarizes the results of the Vitotox-384 test for 61 NTP compounds. Twenty-six Ames-positive compounds gave positive results in the Vitotox-384 test. Styrene oxide, 1,2-epoxybutane and 8-hydroxyquinoline gave negative results in the Vitotox-384 assay, as indicated in the Vitotox-96 assay [1]. We could not examine sufficient higher dose levels for styrene oxide and 1,2-epoxybutane because these chemicals causticized the plastic microplates used. It was reported that 8-hydroxyquinoline is not genotoxic in the SOS chromotest and the *umu* test [5]. Three compounds (2,4-diaminotoluene, 2,6-diaminotoluene and 1,2-dimethylhydrazine) gave inconclusive results because these compounds enhanced a light emission not only in the Genox strain but also in the Cytox strain.

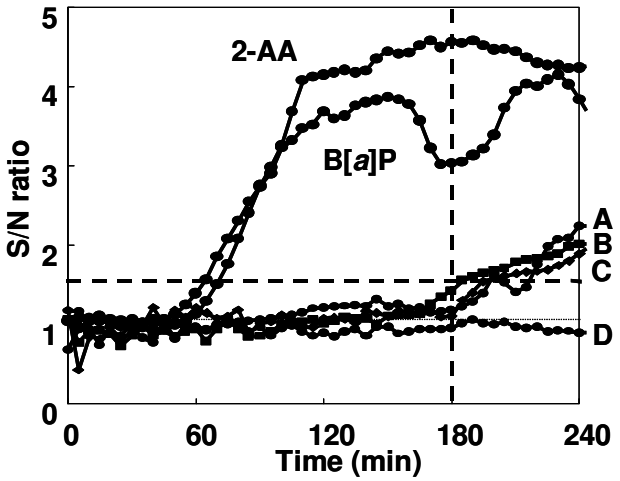
Twenty-five of 28 Ames-negative compounds gave negative results in the Vitotox-384 test. Three compounds, caprolactam, isophorone and thioacetamide, gave inconclusive results, because they enhanced light emission in both Genox and Cytox strains.

The sensitivity of Vitotox-384 for NTP selected compounds was about 87% (26/30, excluding three inconclusive results); negative specificity was 100% (25/25, excluding three inconclusive results). The concordance between the Vitotox-384 test and the Ames test was about 92%.

We found that Vitotox test sensitivity could be improved by modification of the treatment time from 180 min, specified by a manufacturer, to 240 min. In many cases, the induction of light emission in the Genox strain by genotoxins start at about 60 min after incubation and reaches a maximum within 180 min. However, we have experienced that some compounds showed “delayed enhancement” of light emission in the Genox strain. These chemicals can be detected as genotoxic only with a prolonged

treatment (Fig. 1). This fact indicates the advantage of kinetic analysis in the Vitotox test over end-point assays like the *umu* test.

a)



b)

Compound	Vitotox (+S9)		Ames (+S9)
	3h	4h	
A	-	+	+
B	-	+	+
C	-	+	+
D	-	-	-

Fig. 1 The "delayed induction" of light emission in the Genox strain.

a) Kinetics of light emission in the Genox strain. b) Results of the Vitotox test with different incubation times.

We have already screened more than a thousand chemicals synthesized in Mitsubishi Pharma Corporation for genotoxicity since 2001 with the Vitotox-384 test. A total of 137 compounds were tested with both the Vitotox-384 test and Ames test (Table 2). The sensitivity of the Vitotox-384 test for the Ames–positive compounds is 72.4% (21/29). Six of the eight chemicals that gave false negatives in the Vitotox-384 test belonged to the same medical application category. This might be caused by some

Table 2 Comparison of the results of the Vitotox-384 test and the Ames test for newly synthesized compounds in Mitubishi Pharma Corporation

Ames	Vitotox-384		
	Positive	Negative	total
Positive	21	8	29
Negative	0	108	108
Total	21	116	137

SOS-repair independent mechanism of mutagenicity. All of the 108 Ames-negative compounds gave negative results in the Vitotox-384 test.

Fig. 2 Changes in the rate of positive results in the Vitotox and Ames tests

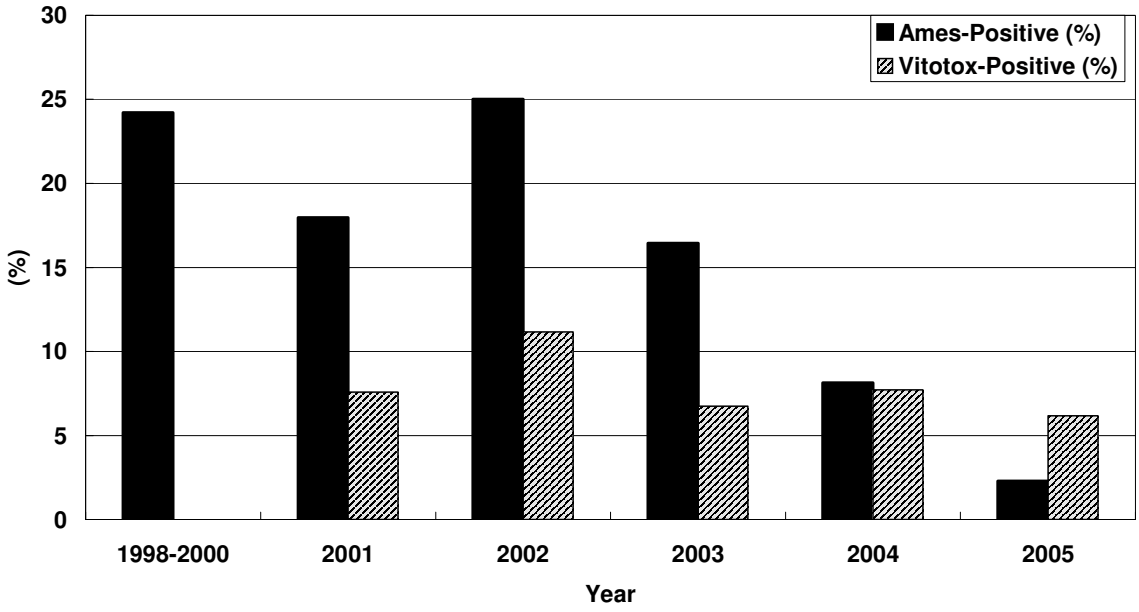


Fig. 2 shows the rate of positives results in the Vitotox-384 test and Ames test for our newly synthesized compounds from 1998 to 2005. We screen newly synthesized chemicals with the Vitotox test first. If the substance gives a negative result with the Vitotox test, we confirm the result with the Ames test at a later stage. If the substance gives a positive result in the Vitotox test, we do not conduct any futher evaluation for the chemical and try to evolve the structure. The positive rates with the Vitotox-384 test have shown constant values of about 7 to 11% over the last four years. In contrast, positive rates with the Ames test have decreased year by year since we introduced the Vitotox-384 test, indicating that genotoxic chemicals are screened out well using the Vitotox test in the early stage. In other words, genotoxic prescreening of genotoxicity with the Vitotox-384 test works well.

The Vitotox-384 test needs less than 10 mg of compound and allows us to test over 15 chemicals/day, even using a manual procedure. The simple test procedure involved may make the test easy to automate to improve the throughput.

In conclusion, the Vitotox-384 test is available for genotoxic screening of newly synthesized chemicals in the early stage of pharmaceutical development.

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