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VITOTOX® Bacterial Genotoxicity and Toxicity Test for the Rapid Screening of Chemicals

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The VITOTOX® test is a new bacterial genotoxicity test that was previously shown to be very rapid and sensitive. Initially only one *Salmonella typhimurium* strain (TA104 *rec*N2-4) was used in the test. In this paper we introduce a second strain (TA104*pr1*) that can be used as an internal control to further enhance the reliability of the test. We demonstrate the usefulness of this *pr1* strain in genotoxicity and toxicity testing. We also report on the results of a study where the VITOTOX® test was performed on newly synthesized pharmaceutical compounds, or intermediate products in the synthesis of drug candidates. We demonstrate that the test gives identical results when performed independently in two different laboratories and that it correlates well with either the Ames test or SOS chromotest. Environ. Mol. Mutagen. 33:240–248, 1999 © 1999 Wiley-Liss, Inc.

Key words: VITOTOX® test; genotoxicity; toxicity; TA104 *rec*N2-4; TA104*pr1*

INTRODUCTION

We recently reported a new bacterial genotoxicity test which is based on bioluminescence and allows an easy, very rapid, and inexpensive detection of genotoxic compounds. The test was shown to be at least as sensitive as the Ames test and SOS-chromotest and to allow genotoxicity kinetics measurements as well as a simultaneous evaluation of the toxicity of the test compound or material [van der Lelie et al., 1997]. This new test, referred to as the VITOTOX® test, was therefore considered to be a valuable short-term (geno) toxicity test for many different purposes.

The test is based on bacteria that contain the *lux* operon of *Vibrio fischeri* under transcriptional control of the *rec*N gene, which is part of the SOS-system. After incubation of the bacteria in the presence of a genotoxic compound, the *rec*N promoter is derepressed, resulting in expression of the *lux* operon. This expression results in light production in function of genotoxicity. Originally, the test was performed with different modified *Escherichia coli* and *Salmonella typhimurium* strains. *Salmonella typhimurium* strains (TA98, TA100, TA102, and TA104) were further used, as the bacteria are well-known for mutagenicity testing and because the same bacteria could also be used for a classical Ames test, should this be required. The construct using a *rec*N promoter up mutation (recN 2–4) gave the best results in all strains. Furthermore, as all Salmonella strains gave very comparable results, we decided to use only the TA104 construct (called TA104 (*rec*N2–4)), as it was shown to be sometimes more sensitive than the other hybrid strains [van der Lelie et al., 1997].

As it was realized that some compounds act directly on the light production (e.g., aldehydes) or enhance the metabolism of the bacteria creating false-positive results, we also introduced a constitutive light-producing strain with a *lux* operon under control of the strong promoter, *pr1*. This is used as an internal control system.

In this paper we report on the use of the constitutive light-producing *pr1* strain to improve the VITOTOX® test as a genotoxicity *and* toxicity test.

Furthermore, as screening for genotoxic compounds is very important in the pharmaceutical industry, a prevalidation study was undertaken in which a number of initially newly synthesized intermediates were tested. All compounds were synthesized at the Janssen Research Foundation (Beerse, Belgium) and tested for their genotoxic properties by either the classical Ames test [Maron and Ames, 1983] and/or SOS-chromotest [Quillardet and Hofnung, 1993], and by the VITOTOX® test. The purpose of this study was to determine the robustness of VITOTOX® test

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relative to other genotoxicity tests in screening of molecules synthesized during the process of new drug development.

MATERIALS AND METHODS

Ames Test and SOS-Chromotest

The SOS-chromotest and the Ames test are well-known and widely used bacterial genotoxicity tests [e.g., Quillardet and Hofnung, 1993; Mersch-Sundermann et al., 1994; Mortelmans et al., 1986]. The "classical" Ames test was routinely performed with *Salmonella typhimurium* strains TA98 and TA100, using the standard protocol described by Maron and Ames [1983]. The SOS-chromotest was purchased as a test kit from Orgenics (Yavne, Israel). The test was performed as indicated in the manufacturer's instructions.

The VITOTOX® Test

Salmonella typhimurium strains

The *rec*N promotor region of *E. coli* [Rostas et al., 1987] contains two LexA binding sites. One LexA binding site overlaps with the -35 region, while the second overlaps with the -10 region and the transcription start point of the *rec*N promoter. The *E. coli rec*N promoter was cloned upstream of the *lux*CDABE operon into the expression vector pMOL877, yielding pMOL1066. Expression of the *lux* operon in this construct is SOS-regulated, resulting in light production when strains harboring this construct are treated with the genotoxins that induce SOS. Some *rec*N promoter derivatives were also cloned into pMOL877. One, lacking the LexA2 site, was pMOL1067, another containing a promoter up mutation was pMOL1068, and a third, lacking both the lexA site and containing the promoter up mutation, was pMOL1069. All constructs were introduced into the Ames test strains TA98, TA100, and TA104 and were able to detect genotoxic compounds. However, as the best results were obtained with strain TA104 (pMOL1068), this strain was used in the VITOTOX® test. It was extensively described before and was designated as TA104 *rec*N2–4 as it contains the *rec*N2–4 PCR fragment [van der Lelie et al., 1997]. Besides TA104 *rec*N2–4 (the tester strain), the TA104 *pr1* strain is also used as a "control strain." Plasmid pMOL 1046 was constructed by random cloning of *Eco*RI-digested DNA fragments from *Alcaligenes eutrophus* CH34 in the *lux*CDABE expression vector, pMOL877. *A. eutrophus* CH34 is a Gram-negative nonpathogenic soil bacterium derived from a site heavily polluted with heavy metals. After transformation into *E. coli*, clones were selected for light production. The best constitutive light-emitting clone was then selected out of the different plasmid transformants (= plasmid pMOL1046) and introduced into the *S. typhimurium* strain, TA104. This was named the "*pr1*" strain. It contains *lux*-genes under control of a constitutive promoter so that the light production is not influenced by genotoxic compounds. The *pr1* strain is used in parallel with the *rec*N2–4 strain and is cultivated and treated in exactly the same way.

Test Procedure

Cultures. Bacteria were incubated overnight on a rotative shaker at 37°C in a *normal bacterial growth medium* supplemented with extra $CaCl_2$ to allow optimal bacterial growth. The next morning, the bacterial suspension was diluted 10 times in medium, and 50 μl of the dilution were then inoculated in 2.5 ml of the medium and incubated for one more hour at 37°C on a rotative shaker (170 rpm).

Preparation of the 96-well plates. Ninety-six-well plates were prepared so as to contain 10 μl of either the solvent, different concentrations of the test compound, or the positive control for genotoxicity testing with (2-AF) or without (4-NQO) S9-mix. All solvents used so far (water, DMSO, ethanol, and methanol) proved suitable in the VITOTOX® test. A single well was used per concentration or replicate. The S9-mix was prepared freshly before use. For tests with S9-mix, 140 μl of the bacteria (*rec*N2-4 or *pr1*) were added to 860 μl medium and 400 μl S9-mix. From this mixture, 90 μl were then added to the 10-μl solution already present in the wells. For tests without S9-mix, 1,260 μl growth medium were added to 140 μl of the bacterial suspension, and 90 μl of the mixture were then transferred to wells containing 10 μl of the test compound or controls.

Genotoxicity and toxicity measurements. A 96-well microplate luminometer (Ultrafast Photon Counter from EG & G Berthold, Vilvoorde, Belgium) was used for measurements of light production following exposure to the test compounds. Light emission from each of the wells was measured every 5 min over 5 hr (30°C, 1 sec/well, 60 cycles of 300 sec each). After completing the measurements, the data were transferred into an Excel (Microsoft, Redmond, WA) macrosheet and the signal-to-noise ratio (S/N), i.e., the light production of exposed cells divided by the light production of nonexposed cells, was calculated for each measurement. A compound was considered genotoxic when the S/N was higher than 2 for at least two concentrations and when a clear dose-dependent relationship was observed.

In experiments where strain TA104 *pr1* was used, the S/N was calculated for the *Rec*N2–4 and pr1 strains separately, as well as the ratio between the maximum S/N values of the *rec*N2–4 and *pr1* strains (*rec/pr1*). All calculations were based on measurements made between 60–240 min of incubation. Here, a compound is considered genotoxic when max S/N (recN2–4)/max S/N (pr1) >1.5 (limit set on experimental grounds). In this way "false positives" can be avoided. An example is given in Table I. Criteria for deciding whether a compound is genotoxic are as follows:

a) The maximum signal-to-noise ratio in the recN-strain must show a good dose-effect relationship.
b) There must be a dose-response relationship in max S/N (recN2–4)/max S/N (pr1), and this should attain a value greater than 1.5.
c) If S/N increases very quickly during the first 20 min, one may not consider it as a genotoxic effect (SOS takes at least 20 min to start). Note: in such a case, the maximum S/N is reached most of the time within 1 hr and shows a descending trend after this time.
d) If both strains are strongly induced, one may not conclude genotoxicity, even when rec/pr1 >1.5.
e) If the maximum S/N for the recN2–4 strain is below 1.5, the result is negative even when rec/pr1 >1.5.
f) If S/N is rapidly decreased below 0.8, there is a toxic effect.

Previous experiments demonstrate that results of independent experiments were highly reproducible (see Fig. 1).

The *pr1* strain is valuable in evaluating toxicity. Toxicity is assumed when the light emission is substantially *decreasing* in a dose-dependent way and attains S/N values lower than 0.8.

Test Compounds

A number of commercially available, well-known compounds that were evaluated previously with the TA104 *rec*N2–4 strain alone [see van der Lelie et al., 1997] were reevaluated in the present work using the TA104 *rec*N2–4 *and* TA104 *pr1* strain. They are given in Table II.

Other compounds that were synthesized at the Janssen Research Foundation (Beerse, Belgium) were included in the present comparative study. For reasons of confidentiality, Table II only gives, as an example, these compounds from which the chemical description can already be given.

The highest concentration of a test material used was test compound-dependent, but was generally chosen at the limit of solubility.

RESULTS

In the VITOTOX® test, light is measured at given time intervals (e.g., every 5 min), and this during a given period of time (e.g., 4 hr; see Fig. 2). Earlier reported results with

TABLE I. Example of a Number of Consecutive Relative Luminescence Values (5-min Intervals) Obtained in Unexposed and 128-ppm MMS Exposed *Rec*N2–4 and *pr1* Salmonella Strains Together With Their Respective Signal to Noise Ratios[a]

RecN2–4			pr1		
0 ppm	128 ppm	S/N	0 ppm	128 ppm	S/N
531	8,033	15.11857	6,061	13,785	2.274252
555	8,744	15.76442	6,438	14,819	2.301802
586	9,539	16.2689	6,825	15,951	2.337029
605	10,313	17.03689	7,137	17,145	2.40227
644	11,068	17.18634	7,524	18,344	2.438065
660	11,881	18.01061	7,841	19,596	2.499277
683	12,729	18.6278	8,240	20,973	2.545164
720	13,655	18.96528	8,635	22,451	2.600000
759	14,611	19.25033	9,110	24,274	2.664447
793	15,623	19.70942	9,704	26,073	2.686922
833	16,636	**19,9792**	10,405	28,016	**2.69255**
900	17,710	19.67778	11,370	30,529	2.685127
981	18,902	19.26155	12,545	32,959	2.627262
1,067	20,056	18.79076	14,005	35,969	2.568236
1,183	21,234	17.94423	15,938	39,615	2.485517
1,308	22,358	17.09327	18,424	43,603	2.366641
1,476	23,640	16.01626	21,472	48,201	2.244865
1,666	24,663	14.80076	25,430	53,675	2.110724
1,915	25,742	13.43996	30,255	60,262	1.991825
2,208	26,720	12.09962	36,105	68,020	1.883967

[a]Max S/N (RecN2–4)/Max S/N (pr1) = 19,9792/2,69255 = 7.42.

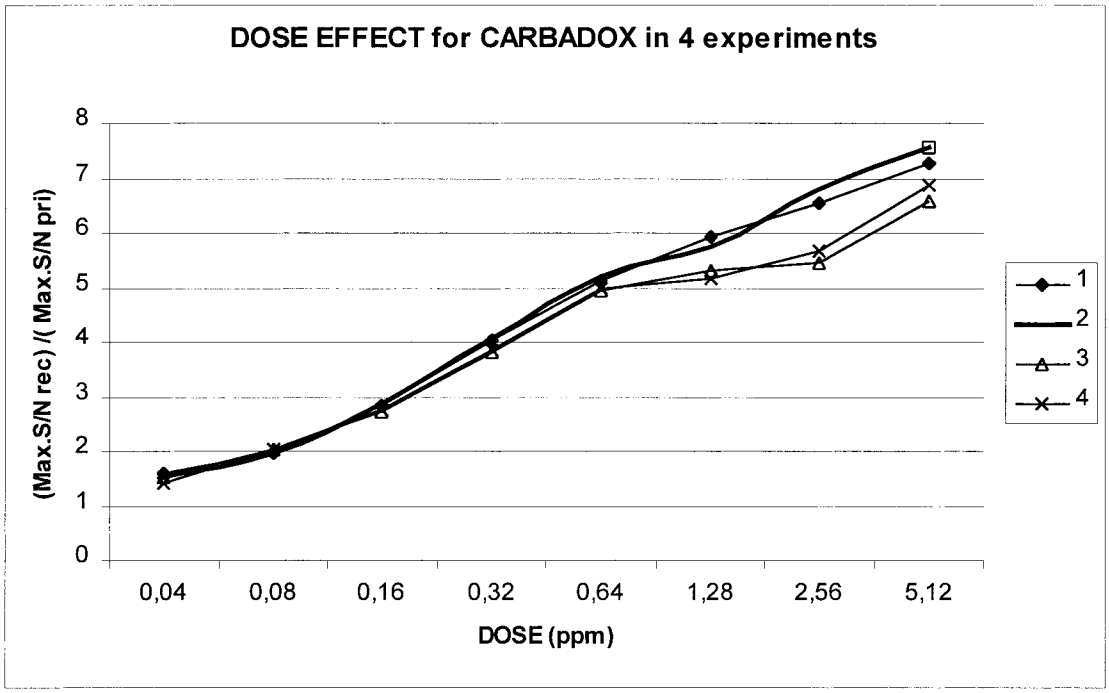

Fig. 1. Results of four independent experiments on carbadox genotoxicity.

the VITOTOX® test were obtained in *Salmonella typhimurium* strain TA104 *Rec*N2–4 [van der Lelie et al., 1997]. Increased light production in treated vs. untreated bacteria was interpreted as a result of genotoxicity. In order to further improve the test we introduced the *pr1 strain*. Now, results obtained in the *RecN*2–4 strain are evaluated in comparison with the results obtained in the *pr1* strain, where an increased light production cannot be due to a genotoxic event. Increased light production in the *rec*N2–4 strain can only be interpreted as an indication of genotoxicity if this is not accompanied by a comparable increase in light production in the *pr1* strain (see Materials and Methods). Table I

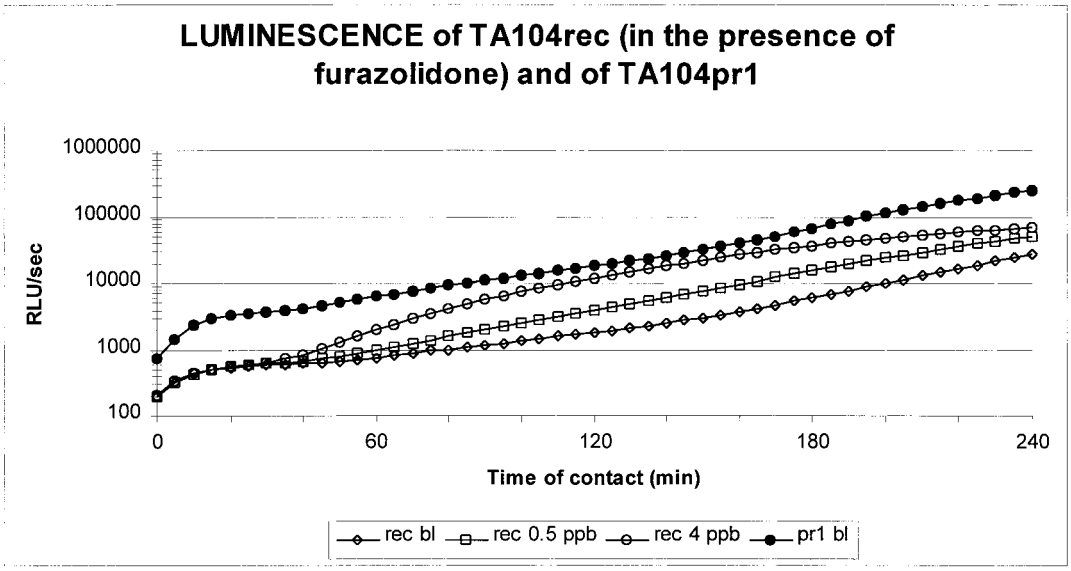

Fig. 2. Luminescence of TA104rec in the presence of furazolidone and of TA104pr1.

gives an example of 20 consecutive measurements around the maximum value for an experiment involving methyl methane sulfonate (MMS). The table illustrates the way the measurements are performed. It gives the values for untreated and treated cultures of the RecN2–4 and pr1 Salmonella strains, together with the obtained signal-to-noise (S/N) ratios. The ratio between the maximum RecN2–4 (S/N) and maximum pr1(S/N) being 7.42, a genotoxic response can be assumed as far as the other requirements indicated in Materials and Methods are fullfilled (e.g., dose-response relationship).

Using both bacterial strains, we reevaluated a number of the earlier studied chemicals. The results are given in Table II. Table II gives the concentrations where the ratio between the maximum *rec*N2–4 S/N and *pr1* S/N (*rec/pr1*) reaches 1.5 or more (minimum detectable concentrations). It also gives the corresponding maximum luminescence values and indicates the presence of toxicity within the dose-range tested and as evaluated by the *pr1* S/N curves. It can be seen that known genotoxic compounds were indeed evaluated as genotoxic, whereas nongenotoxic compounds did not show the required S/N ratios in the given dose-ranges. A few examples of the results are graphically represented in Figures 3–6 (examples of tests without S9-mix). For reasons of clarity we only show 4 doses out of 8 tested. Figure 3 gives the S/N curves for epichlorohydrine in the *rec*N2–4 and *pr1* strains. In the *rec*N2–4 strain, the S/N values became greater than 2 at the dose of 256 ppm, whereas S/N values in the *pr1* strain did not greatly deflect from 1. Figure 4 gives the results for $ZnCl_2$; the results indicate that $ZnCl_2$ is nongenotoxic but is toxic at concentrations higher than 7.4 μM. Sodium azide is given as a third example in Figure 5. Here the S/N ratio was considerably greater than 2 in both the *rec*N2–4 and *pr1* strains, and indications of toxicity were obtained over time (S/N <0.8). Figure 6 illustrates the results that were obtained for nifuroxazide. Referring to the *rec*N2–4 strain, lower doses were apparently more genotoxic than higher doses, but the *pr1* strain showed a dose-dependent decrease in light production, indicating toxicity. Figures 7–10 show some examples of results for compounds requiring S9-mix, whereas Figure 11 gives another example of the results obtained in different independent experiments. It can be seen that the results are very reproducible.

Table III summarizes the results obtained on a number of newly synthesized compounds tested by the different bacterial test systems. Results are expressed as *positive* (genotoxic) or *negative* (not genotoxic). Many more compounds were evaluated and compared in different test systems, but for reasons of confidentiality we cannot yet communicate their chemical composition. From the data presented in Table III, it is apparent that there is good agreement between the results found in the different tests. Yet, some differences were found for the compounds T000836, T001340, and T001409. Compound T000836 was evaluated as "negative" in the SOS-chromotest and the VITOTOX® test, but "positive" in the Ames test, whereas for compound T001340 and T001409, the VITOTOX® results differed with the SOS-chromotest, while agreeing with the Ames test. It should be noted that there was 100% agreement between the VITOTOX® results obtained in the laboratories at the Janssen Research Foundation and at VITO.

DISCUSSION

We demonstrated previously that the VITOTOX® test is a sensitive and rapid method to detect genotoxic compounds [van der Lelie et al., 1997]. However, if only the *rec*N2–4 strain is used (as was initially done), some misinterpreta-

TABLE II. VITOTOX Test Results for Selected Chemicals, with Some Chemicals Investigated Several Times in Different Dose-Ranges or Conditions

Compound	S9[b]	Dose range	MDC	Corresponding RLU values: Rec untreated	Rec treated	pr1 untreated	pr1 treated	S/N rec	S/N pr1	rec/pr1	Toxicity (pr1)
Furazolidone	−	0.125–32 ppb	0.5 ppb	5,290	9,160	12,057	11,829	1.73	0.98	1.76	−
4NQO	−	0.4–102 ppb	0.8 ppb	6,239	10,999	35,513	35,694	1.76	1.01	1.75	−
Nifuroxazide	−	2–256 ppb	8 ppb	7,969	14,430	226,983	243,897	1.81	1.07	1.69	+
MMC	−	3.9–500 ppb	15.6 ppb	13,632	24,876	10,217	10,045	1.82	0.98	1.86	−
3-Nitrofluoranthene	−	7.9–1,000 ppb	15.6 ppb	10,132	16,945	46,403	44,637	1.67	0.96	1.74	−
3-Nitrofluoranthene	+25	7.9–1,000 ppb	15.6 ppb	1,298	2,149	43,089	41,108	1.66	0.95	1.74	−
Nifuroxazide	−	0.04–5.12 ppm	0.04 ppm	8,285	34,270	158,393	153,440	4.14	0.97	4.27	+
3-Nitrofluoranthene	−	4–512 ppb	16 ppb	13,299	19,609	174,597	168,658	1.47	0.97	1.53	−
Carbadox	−	0.04–5.12 ppm	0.04 ppm	16,488	27,033	135,880	137,840	1.64	1.01	1.62	−
Nalidixic acid	−	0.02–2.56 ppm	0.16 ppm	3,267	8,334	16,775	20,389	2.55	1.22	2.10	+
2,4,5,7 Tetranitro-9-fluorenone	−	0.01–1.28 ppm	0.04 ppm	28,786	47,255	101,025	95,309	1.64	0.94	1.74	+
B(a)P	+25	0.025–6.4 ppm	0.2 ppm	11,639	23,999	275,063	279,864	2.06	1.02	2.03	−
2AF	+25	0.2–3.2 ppm	0.2 ppm	4,095	9,413	2,576	2,671	2.30	1.04	2.22	−
B(a)P	+25	0.1–1.6 ppm	0.2 ppm	461	999	2,339	2,519	2.17	1.08	2.01	−
2,7 Dinitrofluorene	+25	0.04–10 ppm	0.62 ppm	19,776	39,689	4,330	4,602	2.01	1.06	1.89	−
B(a)P	+100	0.1–12.8 ppm	0.4 ppm	2,222	3,629	7,414	7,916	1.63	1.07	1.53	−
ICR 191 Acridine	−	0.02–2.5 ppm	0.31 ppm	7,744	12,687	32,658	34,667	1.64	1.06	1.54	−
a-Naphtylamine	+25	0.08–10 ppm	2.5 ppm	20,585	48,615	156,887	206,621	2.36	1.32	1.79	−
4Nitro-o-phenylenediamine	−	0.79–100 ppm	1.6 ppm	8,599	14,506	21,052	22,249	1.69	1.06	1.60	+
Fluoranthene	+100	3.1–400 ppm	3.1 ppm	11,125	23,251	8,663	9,533	2.09	1.10	1.90	−
H2O2	−	0.25–32 ppm	2 ppm	2,039	2,480	21,093	12,078	1.22	0.57	2.12	+
K2Cr2O7	−	0.5–64 ppm	4 ppm	20,816	44,223	30,648	35,095	2.12	1.15	1.86	+
Phenanthrene	+100	3.1–400 ppm	6.2 ppm	12,581	25,558	96,094	100,210	2.03	1.04	1.95	+
MMS	−	4–64 ppm	8 ppm	233	484	823	1,004	2.08	1.22	1.70	−
MMS	−	0.5–128 ppm	8 ppm	8,356	19,396	70,374	75,907	2.32	1.08	2.15	−
Chrysene	+100	0.15–20 ppm	5 ppm	16,492	32,145	83,438	106,589	1.95	1.28	1.53	−
4Nitro-o-phenylenediamine	+25	0.79–100 ppm	12.5 ppm	2,295	5,308	1,830	2,664	2.31	1.46	1.59	+
N-Nitrosodiethylamine	+25	3.25–480 ppm	240 ppm	3,074	9,494	6,871	12,533	3.09	1.82	1.69	−
Epichlorohydrine	−	4–512 ppm	128 ppm	5,271	9,216	12,066	12,241	1.75	1.01	1.72	−
EMS	−	8–1,024 ppm	256 ppm	4,491	10,993	6,158	7,921	2.45	1.29	1.90	−
Epichlorohydrine	−	8–1,024 ppm	128 ppm	14,859	22,972	16,980	16,485	1.55	0.97	1.59	+
ZnCl2	−	0.5–64 ppm	−	−	−	−	−	−	−	−	+
CdCl2	−	0.78–100 ppm	−	−	−	−	−	−	−	−	+
Coumermycine A1	−	1.56–200 ppm	−	−	−	−	−	−	−	−	+
Sodiumazide (NaN3)	−	2–256 ppm	−	−	−	−	−	−	−	−	+
2,7Dinitrofluorene	−	0.04–10 ppm	−	−	−	−	−	−	−	−	−
a-Naphtylamine	−	0.08–10 ppm	−	−	−	−	−	−	−	−	−

[a]MDC, minimal detectable concentration (rec/pr1 > 1.5).
[b]μl/ml of S9-mix used at incubation.

tions were possible. This is why we now use concurrently the *pr1* strain. The added value of the *pr1* strain is illustrated by a few examples. In figure 3 an example is given of a genotoxic compound (epichlorohydrine) that was not toxic in the given dose-range. Based on the results obtained from the *rec*N2–4 strain alone, we previously correctly concluded that the compound was genotoxic, as a dose-dependent increase in light production was observed that exceeded the "noise" value by more than a factor of 2 (S/N >2). Inclusion of the *pr1* strain only confirmed this evaluation. If, for example, an increased light production was found in the *pr1* strain, we should conclude that this was due to an induction mechanism other than genotoxicity (e.g., increased cell proliferation which would enhance the "noise level" compared to that of unexposed cultures). This, however, was not the case. There was also no sign of toxicity, as there was no decreased light production. In contrast, this was clearly the case for $ZnCl_2$, as indicated by the curves of Figure 4. The tested dose of 3.7 μM (not shown in Fig. 4) was neither genotoxic nor toxic, but at higher doses a

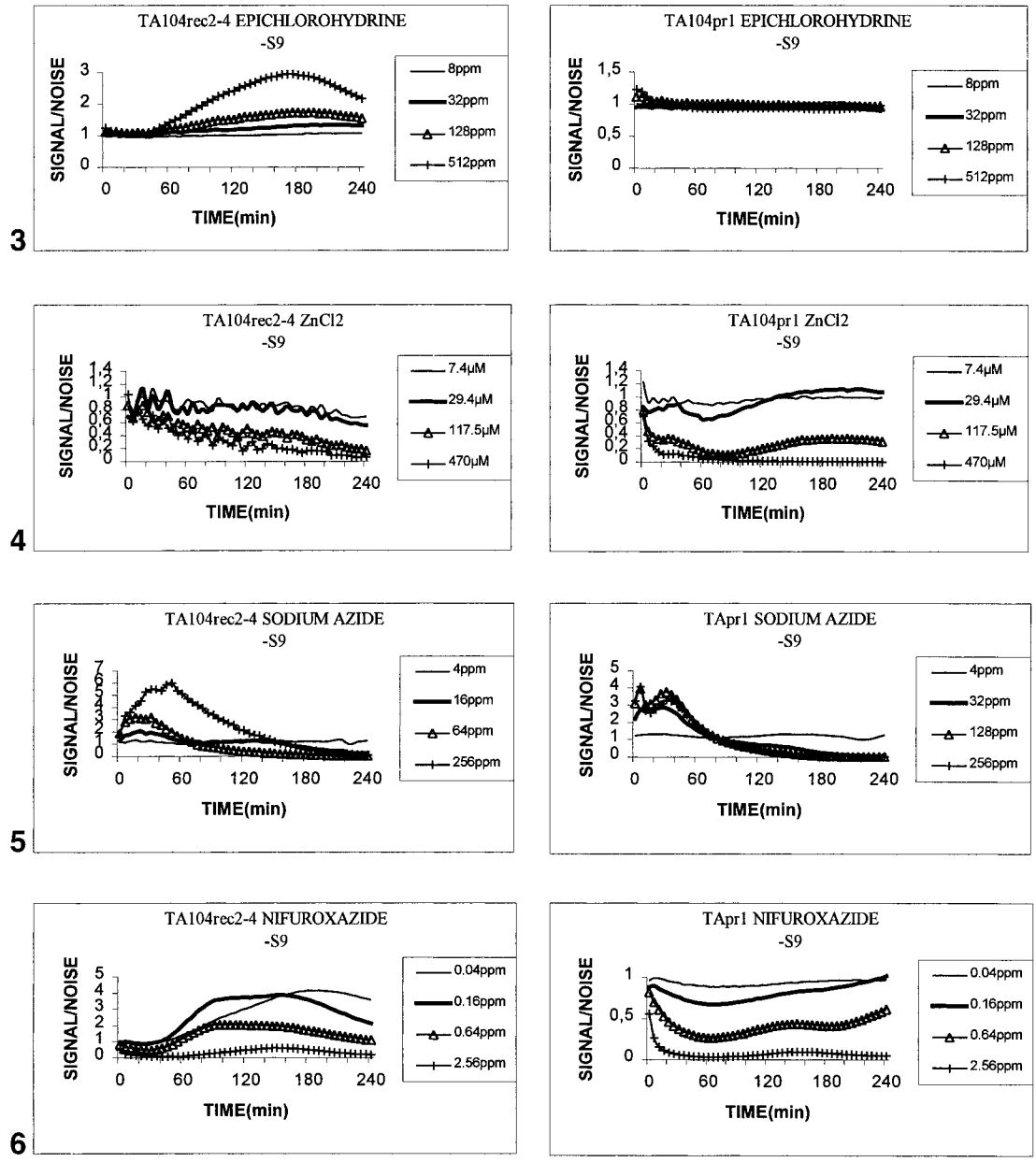

Fig. 3. Signal-to-noise ratio for epichlorohydrine in the *rec*N2–4 and *pr1* strains.
Fig. 4. Signal-to-noise ratio for $ZnCl_2$ in the *rec*N2–4 and *pr1* strains.
Fig. 5. Signal-to-noise ratio for sodium azide in the *rec*N2–4 and *pr1* strains.
Fig. 6. Signal-to-noise ratio for nifuroxazide in the *rec*N2–4 and *pr1* strains.

decreased light emission was observed, indicating a toxic effect. This was confirmed by the *pr1* strain, where some recovery was observed at lower doses. Thus, we conclude that $ZnCl_2$ is nongenotoxic, but at doses above 3.7 µM was toxic in this assay.

As indicated in the Introduction and in Materials and Methods, the VITOTOX® test is based on detection of an SOS signal. It should therefore theoretically produce results that are more in agreement with the SOS-chromotest than with the Ames test. Yet, some differences were previously found. We reported, for example, a "positive" response for sodium azide, although this compound normally scores "negative" in the SOS chromotest [van der Lelie et al, 1997]. One reason for the departure from the SOS-chromotest results might be that the increased light production as found in the *rec*N2–4 strain is due to an induction mechanism other than SOS. With the introduction of the *pr1* strain, we were able to verify this assumption. As seen in Figure 5, the observed light production in the *rec*N2–4 strain was not due to SOS, as increased light emission was also observed in the *pr1* strain. Increased light

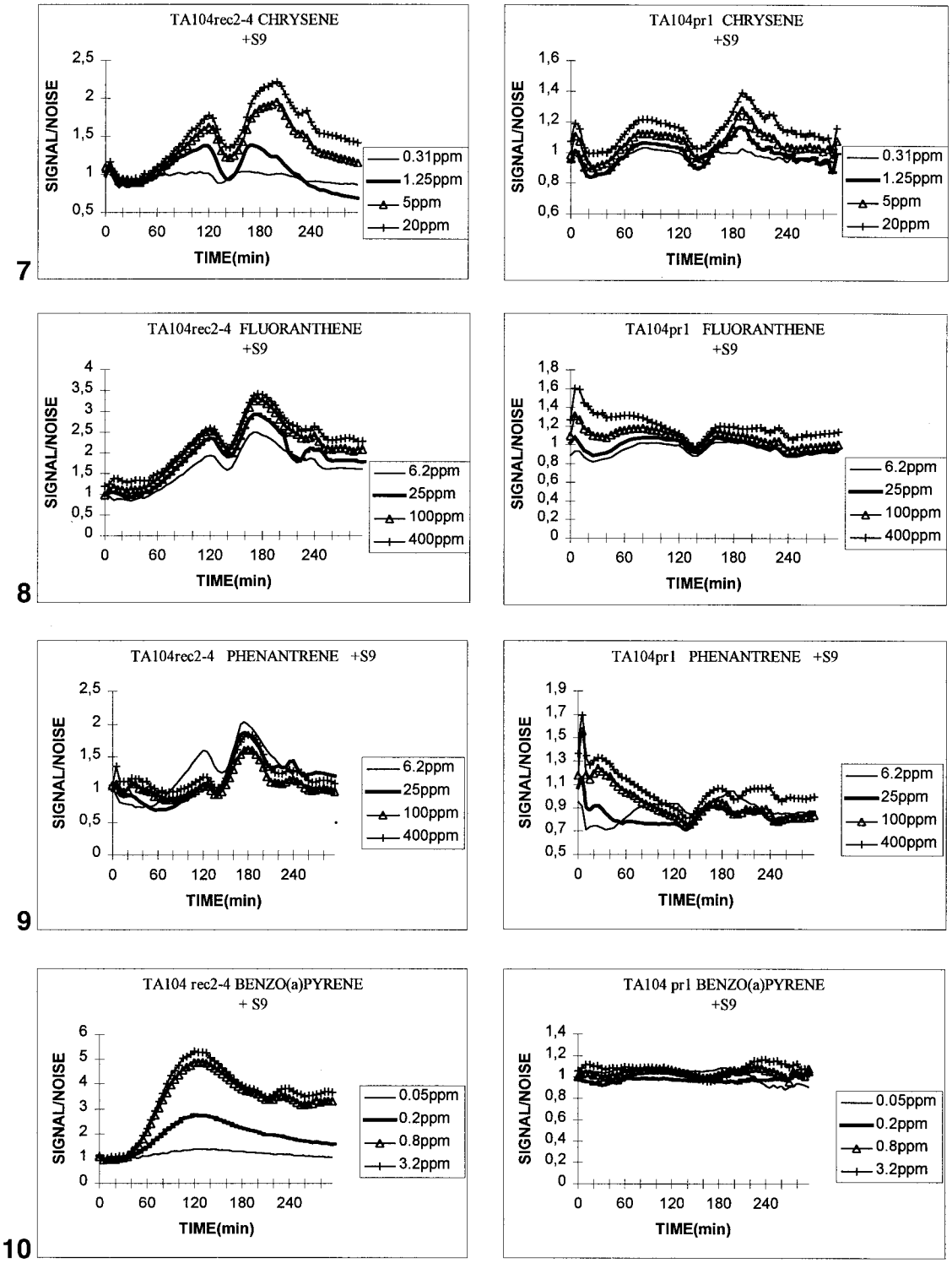

Fig. 7. Signal-to-noise ratio for chrysene in the recN2–4 and *pr1* strains.
Fig. 8. Signal-to-noise ratio for fluoranthene in the recN2–4 and *pr1* strains.
Fig. 9. Signal-to-noise ratio for phenantrene in the recN2–4 and *pr1* strains.
Fig. 10. Signal-to-noise ratio for benzo[a]pyrene in the recN2–4 and *pr1* strains.

production also "starts" earlier than expected for an SOS-regulated response. Sodium azide should therefore be interpreted as a non-SOS-inducing agent in our test. It is an example of a "compound" that might be a "false positive" if the *rec*N2–4 strain alone was used. The chemicals T001340 and T001409, where the SOS-chromotest responded differently

TABLE III. Results of Different Bacterial Genotoxicity Tests Applied to Some Intermediate Compounds

Code	Chemical name	Metabolic activation	SOS-chromotest	Ames test	VITOTOX® (VITO)	VITOTOX® (Janssen Ph.)
T000063	Cyclopropyl(4-fluorophenyl) methanone	−S9	ND	−	ND	−
		+S9		−		−
T000268	(±)-*trans*-3-methyl-1-[(4-methylphenyl)sulfonyl]-4-phenyl-4-piperidine-carbonitrile	−S9	−	−	−	−
		+S9	−	−	−	−
T000407	N-(2-chloroethyl)-N-(1-methylethyl)-2-propanamine hydrochloride	−S9	ND	+	ND	+
		+S9		+		+
T000408	1-(2-pyridinyl)piperazine	−S9	−	−	−	−
		+S9	−	−	−	−
T000836	N-[dihydro-3,3-diphenyl-2(3H)-furanylidene]-N-methylmethanaminium bromide	−S9	−	+	−	−
		−S9	−	+	−	−
T000988	Ethyl 4-[2-amino-4-chlorophenyl)amino]-1-piperidinecarboxylate	−S9	−	−	−	−
		+S9	−	−	−	−
T001326	4-[4-(4-methoxyphenyl)1-piperazinyl]benzenamine	−S9	ND	−	ND	−
		+S9		−		−
T001340	3-bromo-1-(phenylmethyl)-4,4-piperidinediol hydrobromide	−S9	−	+	+	+
		+S9	−	+	+	+
T001409	1,3-Dichloro-2-methoxy-5-nitrobenzene	−S9	−	+	+	+
		+S9	−	+	+	+
T001433	Diethyl (1,3-dioxo-1,3-propanediyl)biscarmate	−S9	−	−	−	−
		+S9	−	−	−	−
T001447	6-Fluoro-3,4-dihydro-2-oxiranyl-2H-1-benzopyran	−S9	ND	+	ND	+
		+S9		+		−
T001866	Methyl 4-(acetylamino)-3-bromo-5-chloro-2-hydroxybenzoate	−S9	ND	+	ND	+
		+S9		−		−

from the VITOTOX® test, may eventually behave like sodium azide. At the time that these compounds were tested, the *pr1* strain was not yet available to us. Unfortunately, it was not possible to reevaluate these chemicals, and thus we are unable to adequately interpret these results. These data do illustrate, however, the added value of the *pr1* strain in the VITOTOX® test. It is also very interesting to consider compound T000836. This compound was indeed *positive* in the Ames test but *negative* in the VITOTOX® test and SOS-chromotest. Among the compounds tested, T000836 appears to be one of the rare compounds that did not show alerts (toxophores) for genotoxicity and carcinogenicity by DEREK, a knowledge-based expert computer system (LHASA Limited, Leeds, UK). Therefore, in this particular case, the VITOTOX® (and SOS-chromotest) results seem to indicate that this compound does not induce SOS.

In using the *pr1* strain, toxicity can be better evaluated than with the *rec*N2–4 strain. The *pr1* strain will clearly show a decrease in light emission, indicating that the compound is toxic at a given dose. This is, at least for some doses, illustrated in Figure 6 for nifuroxazide. The S/N curves for the *pr1* strain clearly indicates that only the lowest dose was not toxic. Higher doses may show toxicity combined with genotoxicity, or may be too toxic to show genotoxicity (highest doses).

The *pr1* strain may provide a tool for those interested in toxicity assessment alone. We are at present comparing toxicity assessments of chemicals and complex mixtures with the *pr1* strain and with the Microtox® test. The latter is one of the most currently used and internationally accepted microbial toxicity tests that is also based on bioluminescence (Hasting, 1978; Férard et al., 1983). According to the limited data available to us, the VITOTOX® test gives similar results to those of the Microtox® test (Microbics, Carlsbad, CA), though the former is easier to perform and is often more sensitive (unpublished results). The *pr1* strain may be a valuable toxicity test if these preliminary results can be confirmed.

In conclusion, it can be stressed that the TA104 *rec*N2–4 and TA104 *pr1 Salmonella typhimurium* strains provide very valuable genotoxicity and/or toxicity test systems. Both strains should be used concomitantly for genotoxicity testing, whereas the *pr1* strain is only required for toxicity testing. It was shown that the VITOTOX® test provides a very rapid (within 2–4 hr) and very sensitive answer with regard to the (geno)toxicity of chemicals, and it may for that reason be very useful in screen-

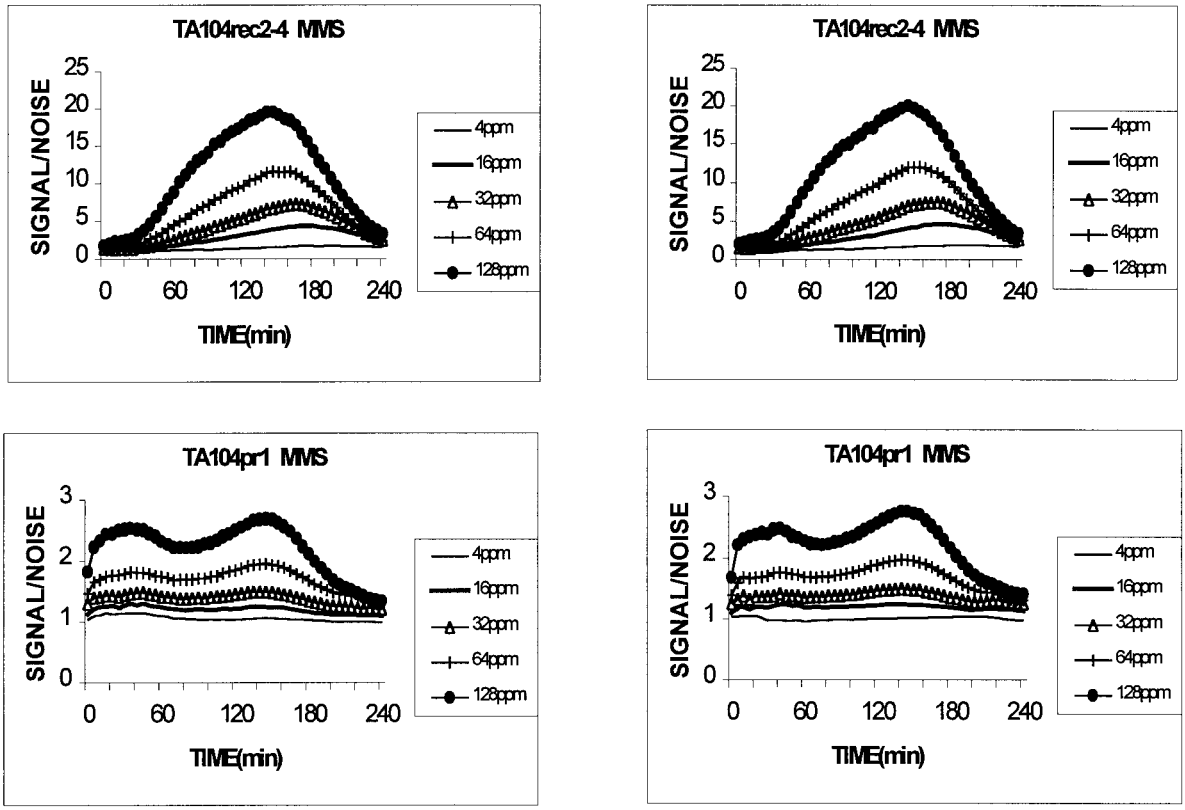

Fig. 11. Results of two independent experiments on MMS.

ing and prescreening of new chemicals and intermediate products. As testing is performed in 96-well plates, it is at least possible to investigate eight chemicals (with and without addition of a metabolic enzyme fraction) per day or 40 chemicals per week. Adaptation of the test for high-throughput screening can be envisaged. We already use 384-well plates in a Labsystems Luminoskan Luminometer (Labsystems Oy, Helsinki, Finland), enabling 4 or 8 times more tests per run.

Measurements occur automatically, and data collection and data handling can also be completely automated, thus reducing labor costs.

Finally, a supplementary and very important asset is that only very small volumes of the test compound are required (less than 20 mg). This is particularly important for the pharmaceutical industry, where only a few hundred milligrams of a compound are available in the *discovery phase* of pharmaceutical development.

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