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A genotoxicity test system based on p53R2 gene expression in human cells: Assessment of its reactivity to various classes of genotoxic chemicals

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ABSTRACT

The tumor suppressor, p53, plays an important role in DNA damage repair, by regulating the expression of target genes. One p53-target gene, *p53R2*, which encodes a subunit of ribonucleotide reductase, is activated by DNA damage. We have previously developed a genotoxicity test system, using human cell lines and a p53R2-dependent luciferase reporter gene assay. 80 chemicals have been examined with this system and 40 of 43 Ames-positive chemicals induced luciferase activity. Eight Ames-negative genotoxic chemicals also induced luciferase activity. Although this assay system could, potentially, be applied to the rapid screening of chemicals that are potentially genotoxic to humans, the ability of the assay to detect genotoxic effects was unclear. In this study, to evaluate the performance of this assay system, several different types of DNA damaging agents were screened. 27 chemicals, whose genotoxic mechanisms are well known, were screened. All genotoxic compounds, except for anti-metabolites and histone deacetylase HDAC inhibitors, showed significant luciferase activity with the following rank order of potency: topoisomerase II inhibitors, intercalaters > bleomycin > topoisomerase I inhibitors > alkylating agents = DNA cross-linking agents = polycyclic aromatic hydrocarbons > spindle poisons. This assay showed greater response to those genotoxic agents that induce DNA double strand break damage compared to those agents that cause other forms of DNA damage. DNA double strand breakage initiates genomic instability, a feature of carcinogenicity. These results indicate that this assay system could be a helpful tool for predicting chemical genotoxicity and carcinogenicity in humans.

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1. Introduction

The p53 tumor-suppressor protein plays a crucial role through its activation of cascades that promote genomic stability, DNA repair, cell cycle arrest, and induction of apoptosis in the response to DNA damage [1–3]. p53 is a transcription factor that binds to specific genomic sites to regulate transcription of downstream multiple target genes [3–5]. p53 is stabilized and activated by post-transcriptional events, such as phosphorylation by the pathway including ataxia-telangiectasia mutated (ATM) kinase/Chks, in response to DNA damage [6]. One of the p53 target genes activated by genotoxic stress is p53R2, which encodes a 351 amino acid protein, a small subunit of ribonucleotide reductase, identified as an hRRM2 homolog and which supplies nucleotides to repair damaged DNA [7,8]. Expression of p53R2 is activated by γ-ray and UV-irradiation, and also by several genotoxic chemicals in a p53 dependent manner [7,9]. p53R2-dependent DNA synthesis plays a pivotal role in cell survival by repairing damaged DNA in the nucleus.

p53-target genes, such as *p53R2*, can, therefore, be markers of genotoxic effects. A consensus DNA-binding sequence for p53, which is a set of two copies of 5′-PuPuPuC(A/T)(A/T)GPyPyPy-3′, was identified in various p53-target genes (Pu; G or A, Py; C or T) [8,11,12]. In a previous report, we constructed a new genotoxicity test system based on a p53R2-dependent luciferase reporter gene assay, using human cell lines and a reporter plasmid containing three tandem repeat sequences of the p53 binding site derived from *p53R2* gene [10]. The advantages of this assay system over conventional *in vitro* mammalian cell genotoxicity tests are as follows: this assay is available for human cell lines that express wild-type p53, and needs only a small number of test samples. In addition, this assay is easier to conduct, is higher throughput, is less time-consuming, and gives a lower false-positive rate compared to conventional tests. In our previous report we examined 80 chemicals. The sensitivity of the assay for Ames-positive chemicals was 93% (40/43) and, the negative specificity of the assay, for Ames-negative chemicals, was 78% (29/37), although differences in

Abbreviations: DSB, double strand break; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; GGR, global genome repair; NER, nucleotide excision repair; BER, base excision repair.

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the intensity of genotoxic effect between this assay and the Ames test were found for each chemical. Those Ames-negative chemicals that resulted in positive assay responses, showed concordance with those of other mammalian genotoxicity tests and carcinogenicity tests in rodents [10].

The mechanism of this genotoxicity test system is through the expression of p53R2, or through the activation of p53 itself as a transcriptional factor, which is unlike other genotoxicity tests. However, the ability of this assay to detect genotoxic effects remains unclear. There are various different classes of DNA damaging agents, which induce different types of genotoxic effects, such as alkylation, DNA cross-linkage, and strand breakage. These genotoxic chemicals are well characterized with respect to the mechanisms of causing DNA damage, and are very helpful tools to evaluate the capabilities of this genotoxic test. Therefore, in order to investigate the performance of this assay system, reactivity to various different types of DNA damaging agents and to non-genotoxic agents that affect the maintenance or metabolism of genome were screened.

2. Materials and methods

2.1. Reagents

All reagents used were of the highest purity grade available. Adriamycin, daunorubicin, camptothecin, cisplatin, benzo[*a*]pyrene, benz[*a*]anthracene, cisplatin, vinblastine sulfate, paclitaxel, methotrexate, 6-mercaptopurine, hydroxyurea, trichostatin A, and butyric acid were purchased from WAKO Pure Chemical Industries, Ltd., Osaka, Japan. *N*-ethyl-*N*-nitrosourea (ENU), methyl methansulfonate (MMS), mitomycin C, vincristine sulfate, nocodazole, and 5-fluorouracil were purchased from SIGMA-Aldrich (St. Louis, MO). *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and cyclophosphamide were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Etoposide, ellipticine, merbarone, and bleomycin were obtained from CALBIOCHEM (San Diego, CA) *N*-methyl-*N*-nitrosourea (MNU) was obtained from Chem Service, Inc. (West Chester, PA). Irinotecan was obtained from Toronto Research Chemicals, Inc. (North York, Canada).

2.2. Cell culture and plasmids

The MCF-7 cell line, derived from American Type Culture Collection (ATCC), was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). MCF-7 cells expressed wild-type p53. Cells were maintained in Eagle's minimum essential medium (MEM, GIBCO, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO), 1% non-essential amino acids (GIBCO), 1 mM sodium pyruvate. Cells were routinely incubated at 37 °C, 5% CO_2, and humidity.

The p53BS-Luc reporter plasmid was constructed by inserting the three tandem repeat sequences for the p53 binding site derived from intron 1 of human *p53R2*, as previously described [10]. pRL-CMV internal control plasmid, encoding the seapansy-derived luciferase gene downstream of the CMV enhancer/immediate early promoter site, was purchased from Promega (Promega, Madison, WI, USA).

2.3. Dose levels

In dual luciferase reporter gene assay, the internal control luciferase activity indicates not only transfection efficacy and cell number but also ability of protein expression [13]. To perform a reporter gene assay in appropriate conditions, the reduction of the internal luciferase activity less than 50%, which can cause false positive results, should be avoid. The test sample concentration where the internal luciferase activity was inhibited by cytotoxic effect was lower than the concentration where the cell viability measured by tetrazolium salts WST-1 in almost all test samples shown in Fig. 1. To determine cell viability, the WST-1 assay was carried out using the Premix WST-1 Cell Proliferation Assay System (TAKARA BIO Inc., Shiga, Japan). MCF-7 cells were plated at an initial concentration of 1.0×10^4 cells/well in 96-well microplates (Corning, Flanklin Lakes, NJ). After 2–4 h incubation, test samples or solvent (final concentration, 0.1%, DMSO) were added. After 22–24 h incubation, microscopic observations were carried out to investigate the precipitation of insoluble test samples, and WST-1 assay was carried out. The highest test concentration of each test compound was determined by its cytotoxic effect (Cell viability is 35–80% in WST-1 assay) and solubility (There is no precipitation of insoluble test sample) as shown in Table 1.

2.4. Luciferase reporter gene assay

MCF-7 cells were plated at an initial concentration of 1.0×10^4 cells/well in 96-well, white, clear-bottomed microplates (Corning, Flanklin Lakes, NJ). The cells in each well were transiently co-transfected with p53BS-Luc plasmid and pRL-CMV plasmid using TransFast™ transfection reagent (Promega) in each assay. 4 h after transfection, medium was changed and test samples or solvent (final concentration, 0.1%, DMSO, total volume 200 μL/well) were added into triplicate wells. After 22–24 h incubation, the cells were washed with phosphate buffered saline (PBS) and lysed by adding 25 μL/well of Picagene lysis buffer solution at room temperature (TOYO B-Net CO., LTD., Tokyo, Japan). Luciferase activities derived from p53BS-Luc and pRL-CMV were measured with a Picagene Dual Seapansy Luciferase Kit (TOYO B-Net CO., LTD.) using a luminometer (Micro Lumat P96V; Berthold, Wildbad, Germany). The Luciferase activity of p53BS-Luc was normalized with that of pRL-CMV as an internal standard. The transfection efficacy of the reporter plasmid was determined by luciferase activity of the seapansy luciferase plasmid. Relative p53R2-dependent luciferase activity after treatment with chemicals was expressed as the % of the control cell activity. Adriamycin (0.125 μg/mL) was used as a positive control in each assay to ascertain the assay working properly. Each assay was triplicated and repeated at least twice to corroborate the results. The inter-

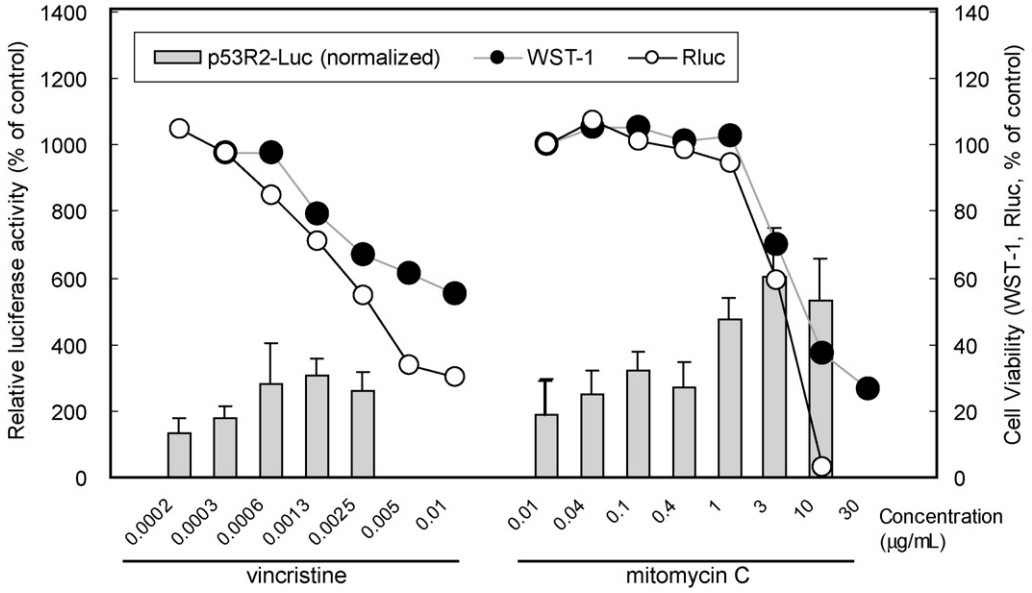

Fig. 1. Cell viability and p53R2-dependent luciferase activity of vincristine and mitomycin C in MCF-7 cells. Cell viability was measured by WST-1 assay (WST-1; closed circle) and internal control luciferase activity (Rluc; open circle). Cells were treated with the indicated concentrations of the test chemicals for 24 h, then WST-1 assay or dual-luciferase assay was carried out. Relative luciferase activity (p53R2–Luc; bar graph) was calculated as described in Section 2.

Table 1
Genotoxicity of chemicals in various *in vitro* genotoxicity tests and in *in vivo* carcinogenicity in rodents

Compounds	CAS no.	p53R2-dependent luciferase[a]		Highest concentration[b] setting		Applicable highest[c] concentration		Published data[d]					
		Result	Highest inducibility (% of control)	μg/mL	Cell viability (% of control WST-1)	μg/mL	Reason	Ames	MLA	CA	MN	SCE	Carcinogenicity in rodents
Topoisomerase II inhibitors and intercalaters													
Etoposide	33419-42-0	+	3707	25	68.8	25	T	–	+	+	+		+
Ellipticine	519-23-3	+	2505	5.0	56.6	2.5	T	+	+		+	+	
Adriamycin	25316-40-9	+	2603	1.0	67.3	0.25	T	+	+	+	+	+	+
Daunorubicin	20830-81-3	+	2725	0.4	79.0	0.05	T	+		+		+	+
Topoisomerase II catalytic inhibitor													
Merbarone	97534-21-9	+	585	50	74.4	25	P	–	+				
Topoisomerase I inhibitors													
Camptothecin	7689-03-4	+	701	1.0	52.1	0.3	T	–	+	+	+		
Irinotecan	100286-90-6	+	605	50	63.6	25	T	–		+			
Radical generator													
Bleomycin sulfate	9041-93-4	+	2555	10	63.1	10	C	+	+	+	+	+	+
Alkylating agents, DNA cross-linkers (or intercalators)													
MNU	684-93-5	+	463	150	74.4	150	T	+	+	+	+	+	+
ENU	759-73-9	+	266	300	50.6	150	T	+	+	+	+	+	+
MMS	66-27-3	+	701	50	51.9	50	C	+	+	+	+	+	+
MNNG	70-25-7	+	522	10	37.9	3.3	T	+	+	+	+	+	+
Cyclophosphamide	50-18-0	+	467	300	63.7	100	T	+	+	+	+	+	+
Cisplatin	15663-27-1	+	466	10	50.0	10	T	+	+	+	+	+	+
Mitomycin C	50-07-7	+	627	3.3	69.7	3.3	T	±	+	+	+	+	+
PAHs, inducing bulky adducts													
Benzo[*a*]pyrene	50-32-8	+	451	3.0	90.0	3.0	P	+	+	+	+	+	+
Benz[*a*]anthracene	56-55-3	+	654	3.0	87.0	3.0	P	+	+				+
Tubulin polymerization inhibitors													
Vinblastine sulfate	143-67-9	+	281	10	74.1	1.0	T	–	+	+	+	+	–
Vincristine sulfate	2068-78-2	+	315	0.01	55.0	0.0025	T	–	+	+		+	I
Nocodazole	31430-18-9	+	531	0.01	61.9	0.01	C						
Microtubule stabilizers													
Paclitaxel	33069-62-4	+	354	75	73.6	8.3	T	–	+	+			
Anti-metabolites													
5-Fluorouracil	51-21-8	–		100	79.2	33	T	–	+	+	+	+	±
Methotrexate	59-05-2	–		100	72.8	100	C	–	+	+	+	+	–
6-Mercaptopurine	50-44-2	–		25	79.8	25	C	+		+	+		–
Hydroxyurea	127-07-1	–		100	69.0	100	T	±	+	+			
HDAC inhibitors													
Trichostatin A	58880-19-6	–		10	62.5	10	C	–					
Butyric acid	107-92-6	–		300	76.3	300	C	–					

Conclusion results for each chemicals: positive, +; negative, –; equivocal, ±; inconclusive, I.

[a] The results of p53R2-dependent reporter gene assay in this study. When the relative luciferase activity of cells treated with the test sample was over 200% of that treated with vehicle only (control cells) in a dose-dependent increasing manner, the test sample was judged to be positive (+) in genotoxicity. Highest inducibility represented the highest relative luciferase activity induced by the test sample as % of control cells.

[b] Highest concentration of test samples to test were determined by its cell viability (indicated as % of control cells) and solubility as described in Section 2. Cell viability, cell viability at the highest concentration tested.

[c] Applicable highest concentrations were determined by: T, Reduction of the luciferase activity of internal control plasmid (less than 50% of control); P, precipitation of insoluble test chemical or C, reduction of cell viability.

[d] Published results in the Ames test (Ames), the mouse lymphoma tk assay (MLA), the in vitro chromosome aberration test (CA), the in vivo/in vitro micronucleus test (MN), the sister-chromatid exchange (SCE), the carcinogenicity in rodents. These data were referred to the published database: Chemical Carcinogenesis Research Information System(CCRIS), Genetic Toxicology Data Bank (GENE-TOX), Carcinogenic Potency Database (CPDB), National Toxicology Program (NTP), the USEPA, or MSDS.

nal control luciferase has a constitutively active promoter driving expression of a second reporter protein. Control reporter activity correlates to the amount of DNA transfected into the cells and the general ability of the cells to express protein. When a chemical-treatment caused seapansy luciferase activity to be less than 50% of the value for control cells, the concentration of the chemical was judged to be "not applicable" for the cytotoxic effect on protein expression. When the relative luciferase activity of cells treated with the test sample was over 200% of that treatment with vehicle only (control cells) in a dose-dependent manner and the difference between luciferase activity of cells treated with the sample and that of control cells was statistically significant, the test sample was judged to be positive for genotoxicity.

2.5. S9 mix

A rat liver S9 fraction, which was derived from livers of 7-week-old male Sprague–Dawley [Crj: CD] rats that had been treated with a combination of phenobarbital and 5,6-benzoflavone, was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Preparation of S9 mix was modified to reduce its cytotoxic effect on MCF-7 cells, according to previous reports [11,12]. 3 μL of the S9 fraction was mixed with 6 μL of 20 mM Hepes buffer (pH 7.2), 42 μL of Co-factor C (Oriental Yeast Co., Ltd.) containing 4 mM Hepes buffer (pH 7.2), 5 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, and 4 mM NADP and 140 μL of culture medium. Test samples (2.0 μL) were added to the S9 mix and incubated at 37 °C for 30 min, and then 20 μL aliquots of the S9 reaction mixture was added to each well.

2.6. Statistical analysis

Values are expressed as the mean ± S.D. We used the Student's *t*-test to determine the statistical significance of between-group differences, with $P < 0.05$ considered as significant.

3. Results

In order to assess the capabilities of this p53R2-dependent luciferase reporter gene assay system, 27 chemicals that induce DNA damage or affect the maintenance or metabolism of the genome were examined.

3.1. Topoisomerase II inhibitors and intercalaters

Topoisomerase II inhibitors and intercalaters were examined (Fig. 2). There are two different topoisomerase II inhibitor mechanisms. Typical topoisomerase II inhibitors, for example etoposide, stabilize cleavable DNA complexes and topoisomerase II, leading to DNA double strand breaks (DSBs). Etoposide and elliptisine induced very high luciferase activity in a dose dependent manner [14,15] (Fig. 2A). Anthracycline antibiotics, adriamycin and daunorubicin, which interact with DNA by intercalation and inhibit topoisomerase II, also induced very high luciferase activity [16,17] (Fig. 2B). Catalytic topoisomerase II inhibitors, such as merbarone, are another type of compound which target topoisomerase II [18]. These drugs act at a point in the enzymes catalytic cycle where DNA breaks do not occur. Merbarone induced luciferase activity in this assay, though the intensity was weaker compared to that of the other topoisomerase II inhibitors (Fig. 2A).

3.2. Bleomycin, an anticancer drug, which generates free radicals

Bleomycin induces DNA strand breaks through the generation of free radicals [19,20]. Bleomycin exhibits intense luciferase activity, similar to topoisomerase II inhibitors (Fig. 2B).

3.3. Topoisomerase I inhibitors

Topoisomerase I inhibitors stabilize the covalent complex of DNA and topoisomerase I, mainly resulting in DNA single strand breaks [21,22]. Camptothecin and irinotecan induced luciferase activity, which was about eightfold higher than that of control cells. The luciferase activity of irinotecan was facilitated by the S9-treatment (Fig. 3).

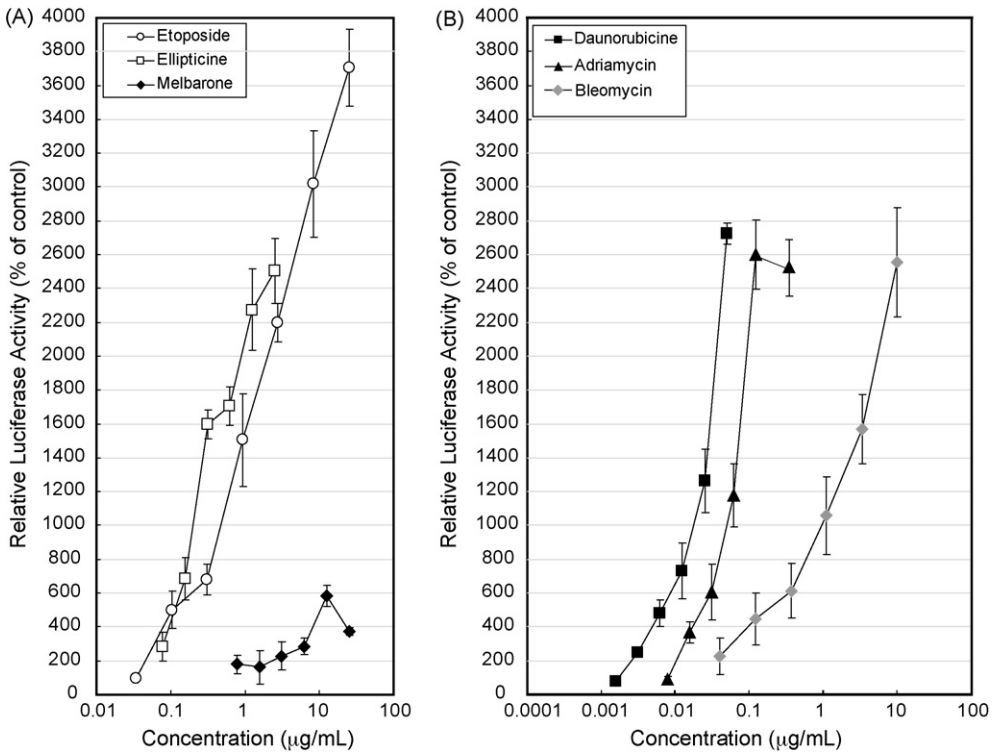

Fig. 2. Effects of (A) topoisomerase II inhibitors, (B) intercalaters, and bleomycin on p53R2-dependent luciferase activity in MCF-7 cells. Cells were treated with the indicated concentrations of the test chemicals for 24 h, then cells were lysed and dual-luciferase assay were carried out. Relative luciferase activity was calculated as described in Section 2. Each value represented the mean ± S.D. of triplicate assays.

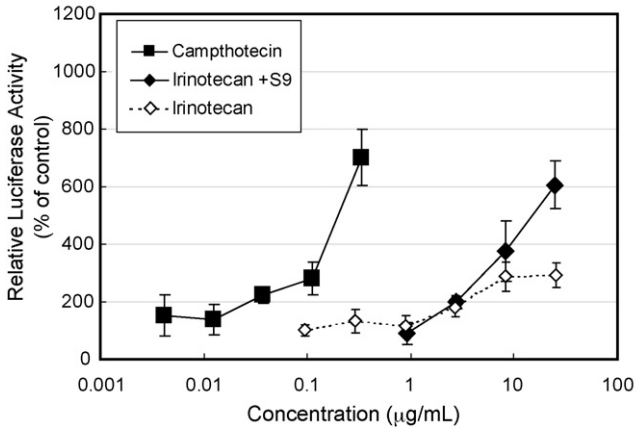

Fig. 3. Effects of topoisomerase I inhibitors on p53R2-dependent luciferase activity in MCF-7 cells. Cells were treated with the indicated concentrations of the test chemicals for 24 h. Relative luciferase activity was calculated as described in Section 2. Irinotecan was treated with S9 mix. Each value represented the mean ± S.D. of triplicate assays.

3.4. Alkylating agents and DNA cross-linkers (or intercalators)

Alkylating agents are chemicals that add an alkyl group to another molecule. Alkylating agents induce miss-pairing of the nucleotides, leading to mutations. Typical alkylating agents, MNU, ENU, MNNG, MMS and cyclophosphamide induced luciferase activity in dose-dependent manners, and the maximum activity was approximately 600% of control (Fig. 4A). Cyclophosphamide treated with S9 mix induced luciferase activity, although it did not show significant induction of the luciferase activity without S9-treatment. Cyclophosphamide needed S9-treatment for genotoxic effect detection in this assay. These results suggested that the S9-treatment could appropriately work.

A bifunctional alkylating agent, mitomycin C, which intercalates into DNA and forms DNA cross-links, induced luciferase activity in a dose-dependent manner (Fig. 4B). Cisplatin also forms DNA cross-links and intercalation of DNA. Mostly it acts on the adjacent N-7 guanine forming 1, 2-intrastrand cross-links. Cisplatin induced luciferase activity in a dose-dependent manner (Fig. 3B).

3.5. PAHs, which form bulky DNA adducts

Polycyclic aromatic hydrocarbons (PAHs), such as benzo(a) pyrene, is metabolized to diol epoxide, which binds covalently to guanine bases [23]. The presence of bulky adducts in the genome increases the error frequency of the replication machinery, causing mutations that contribute to the initiation and progression of cancer. Benzo[*a*]pyrene and benz[*a*]anthracene induced luciferase activity in a dose-dependent manner (Fig. 5A). These chemicals showed higher luciferase activity by the treatment of S9 mix.

3.6. Spindle poisons

There are two different types of chemicals that effect microtubule dynamics. Tubulin polymerization inhibitors, such as vinca alkaloids and nocodazole, block the polymerization of tubulin into microtubules and inhibit cell division [24]. In contrast, Taxanes stabilize microtubule formation and inhibit the cell division [25].

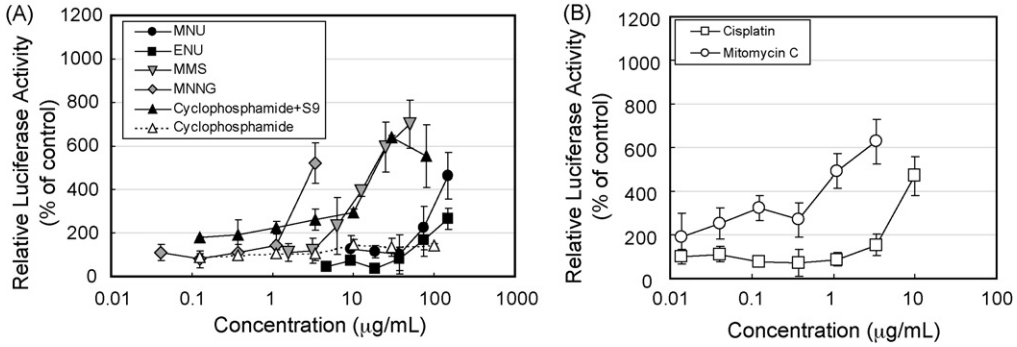

Fig. 4. Effects of (A) alkylating agents and (B) DNA cross-linkers (or intercalators) including a bifunctional alkylating agent on p53R2-dependent luciferase activity in MCF-7 cells. Cells were treated with the indicated concentrations of the test chemicals for 24 h. The relative luciferase activity was calculated as described in Section 2. Cyclophosphamide was treated with S9 mix. Each value represented the mean ± S.D. of triplicate assays.

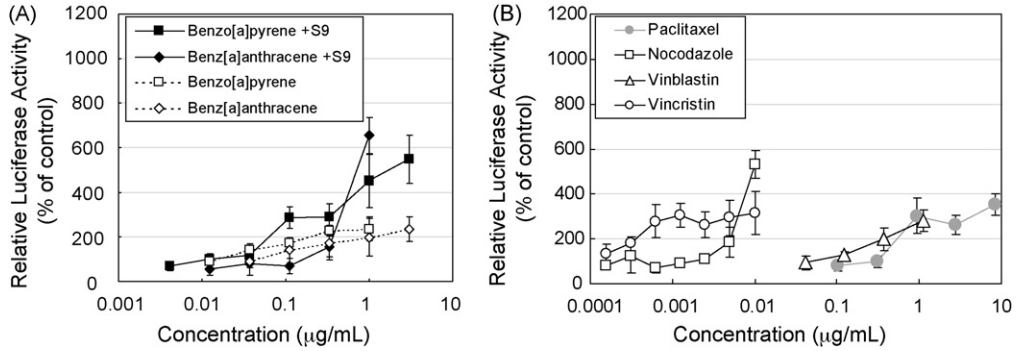

Fig. 5. Effects of (A) PAHs and (B) spindle poisons as the reagents constructing bulky DNA adducts on p53R2-dependent luciferase activity in MCF-7 cells. Cells were treated with the indicated concentrations of the test chemicals for 24 h, then cells were lysed and dual-luciferase assay were carried out. The relative luciferase activity was calculated as described in Section 2. Benzo[*a*]pyrene and benz[*a*]anthracene were treated with S9 mix. Each value represented the mean ± S.D. of triplicate assays.

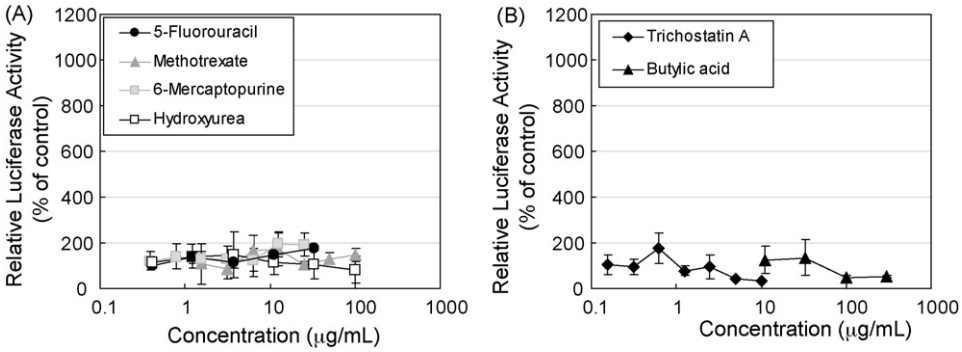

Fig. 6. Effects of (A) anti-metabolites and (B) HDAC inhibitors on p53R2-dependent luciferase activity in MCF-7 cells. Cells were treated with the indicated concentrations of the test chemicals for 24 h. The relative luciferase activity was calculated as described in Section 2. Each value represented the mean ± S.D. of triplicate assays. These test chemicals did not show positive effects.

These chemicals induced significant luciferase activity in a dose-dependent manner. But the activity was low, about only two to fourfold higher than control cells (Fig. 5B).

3.7. Anti-metabolites and HDAC inhibitors

Anti-metabolites, a group of anti-cancer drugs, prevent cells growing and dividing by blocking the chemical reactions required to produce DNA or RNA. Hydroxyurea inhibits ribonucleotide reductase, and interrupts the cell cycle at the G1 and S phases.

Four anti-metabolites, 6-mercaptopurine as a purine analogue, 5-fluorouracil as a pyrimidine analogue, methotrexate as a folic acid analogue, and hydroxyurea were tested. All anti-metabolites tested did not show significant luciferase activity, although 5-fluorouracil, methotrexate, and hydroxyurea induced weak luciferase activity, in a dose-dependent manner (Fig. 6A).

Histone deacetylases (HDAC) are a family of enzymes that catalyze the removal of acetyl groups from core histones, resulting in changes of chromatin structure and gene transcription activity. HDAC inhibitors induce growth arrest, apoptosis, and/or differentiation via transcriptional activation of multiple genes [26,27]. Two HDAC inhibitors, trichostatin A and butyric acid were examined. They show no significant increase in relative p53R2-dependent luciferase activity, and were judged to be negative in this test according to the criteria for determining positive chemicals as described Materials and Methods (Fig. 6B). However both HDAC inhibitors induced not only the luciferase activities derived from p53BS-Luc but also that from the internal control plasmid. The luciferase activities of other internal control plasmids (pRL-SV40, pRL-tk) were also increased in the cells transfected with internal control plasmid only (data not shown). These results could be predicted because HDAC inhibitors do not directly act on DNA but act on enzymes. In addition, their effects on transcriptional activation of multiple genes, induced by the changes of chromatin structure, are thought to be non-specific. Therefore, HDAC inhibitors were negative in this assay.

4. Discussion

The purpose of this study was to determine the ability of a genotoxicity test, p53R2-dependent luciferase reporter gene assay in human cell lines, to detect various classes of genotoxic reagents. To this end, we examined 27 chemicals, which have well characterized mechanisms, including several types of DNA damaging agents and several reagents affecting DNA metabolism or transcription (Table 1). All genotoxic agents, except for anti-metabolites and HDAC inhibitors, induced detectable luciferase activity. The rank order of intensity of p53R2-dependent luciferase activity was: topoisomerase II inhibitors, intercalaters > bleomycin > topoisomerase I inhibitors > alkylating agents = DNA cross-linking agents = PAHs > microtubule stabilizer = tubulin-polymerization inhibitors. Anti-metabolites and HDAC inhibitors did not show significant luciferase activity, although some anti-metabolites induced very weak luciferase activity (<200% of control), in a dose-dependent manner.

There are two pathways in human cells to supply dNTPs for DNA synthesis: one through the activity of hRRM2 for DNA replication during the S phase, and the other through p53R2 for DNA repair in G0–G1 cells [28]. Expression of p53R2, but not hRRM2, is induced by DNA damage. p53R2 is directly regulated by p53 for supplying nucleotides to repair damaged DNA. Therefore, the rank order of the intensity of luciferase activity in this assay is related to the frequency of DNA damage, which activate DNA repair pathway through p53 and p53R2. This p53R2-dependent luciferase assay is the most sensitive to topoisomerase II inhibitors and anthracyclin antibiotics.

Topoisomerase II inhibitors and anthracyclin antibiotics showed the highest luciferase activities. Topoisomerase II inhibitors stabilize DNA cleavage and resulted in induction of DSBs. Anthracycline antibiotics also have topoisomerase II inhibiting activity and intercalate with DNA double strands, and induce DSBs. Bleomycin, which generates free radicals, also induced very high luciferase activity. Some reports suggest that bleomycin chelates metal ions, producing a pseudoenzyme that reacts with oxygen to produce superoxide and hydroxide free radicals that cleave DNA double strands [19,20]. DSBs are lethal forms of DNA damage and initiate genomic instability. They are usually repaired through one of two pathways; end-joining or homologous recombination. Homologous recombination is likely to be the main error-free DNA repair mechanism [29]. DSB miss-repair results in deletion, amplification, and translocation, which are commonly observed in human tumors and are directly implicated in tumour progression [30–32]. Tumor suppressor, p53, is directly involved in responses to DSB damage in human and mouse cells through the post-transcriptional activation including ATM pathway [33–36]. ATM can phosphorylate p53 at Ser15 (corresponding to Ser18 of mouse p53), and this phosphorylation activates p53 responses to DNA DSB damage [37–40]. p53 and p53R2 are well known to be strongly activated by γ-ray irradiation, which induces DSB damage. Therefore, it is very reasonable for this assay to sensitively respond to the genotoxic agents that induce DSB damage. In addition, we suggested that this assay is a useful tool for predicting human carcinogenicity, because of its high sensitivity to DSB.

Topoisomerase I inhibitors stabilize the cleavable complex and thus prevent religation of topo I-mediated DNA single-strand breaks. This gives rise to stabilization and accumulation

of topo I–DNA covalent complexes. Cytotoxicity is triggered by a collision between the replication fork and the camptothecin-stabilized cleavable complex in S phase. This may result in blockage of fork movement, such breaks being the equivalent of a DSB [41]. This double strand break would induce the luciferase activity in this test. It has been reported that cells expressing wild-type p53 show a deficiency to the cytotoxic effect of topo I inhibitor and repress apoptosis, due to the p53 stimulated repair of the topo I inhibitor–topo I–DNA complex [42]. This activation of the p53 pathway by the repair of this complex might itself contribute to increase the luciferase activity in this study. p53 also regulates DNA repair mechanisms through the induction of p53R2, a ribonucleotide reductase subunit, and may also directly participate in repair by promoting annealing of single-stranded DNAs and rejoining double-stranded breaks [43,44].

The alkylating agents tested induced luciferase activity; although the intensities were lower compared to the genotoxic agents that caused strand breaks. Alkylating agents cause many types of DNA lesions, including O6-methylguanine, which results in G:C to A:T transition mutations [45]. This lesion has been shown to induce p53 and apoptosis in normal cells but not in cells lacking the mismatch repair protein complex MutSα [46]. Some alkylating agents stabilized and accumulate p53, not due to the phosphorylation in p53 N-terminus, but to decreased levels of MDM2 mRNA and protein, p53 ubiquitination [47]. This report suggests that the post-translational modification of p53 is not needed to increase p53 levels in the cells treated by the alkylating agents. By contrast, it has been reported that treatment of cells with the DNA alkylating agent MNNG and MMS resulted in phosphorylation of p53 at Ser15 and up-regulation of p53 abundance [48,49]. Further, this phosphorylation event requires functional protein complexes involved in mismatch repair. Though high concentration of MNNG-treatment induces DNA strand breaks, apoptosis-induced DNA strand breaks are not required for the activation of ATM in response to MNNG [50]. But DNA strand breaks that arise during the repair process activate ATM [50]. The base excision repair (BER) pathway can correct DNA base damage generated by alkylating agents [51]. Stimulation of BER by p53 is correlated with its ability to interact directly, both with the AP endonuclease and with DNA polymerase β (polβ) [52]. A DNA cross-linker, cisplatin, induces bulky lesions in DNA and cisplatin adducts in DNA are efficient blocks for transcription both *in vitro* [53] and *in vivo*. The blockage of transcription is sufficient to induce p53 and apoptosis. The DNA damage response induced by cisplatin activates p53 through the ATR-Chk2 pathway [54]. The bulky DNA adducts induced by DNA cross-linker, PAHs, and UV are corrected by nucleotide excision repair (NER). NER recognition and processing of the lesion are required to trigger the signal transduction cascade that leads to the phosphorylation of key checkpoint proteins, such as Chk1 and p53 [55–57]. In addition, p53 transactivates p53R2 [7,8], which is essential for the DNA resynthesis step common to both NER sub-pathways. Thus, these reports suggest that the luciferase activity induced in this assay by alkylating agents, DNA cross-linkers or PAHs were not always due to DSBs.

Spindle poisons, such as tubulin polymerization inhibitors and microtubule stabilizers, which are widely used as anti-mitotic cancer chemotherapeutic drugs, induced luciferase activity. These chemicals inhibit cell division without directly damaging DNA. These chemicals activate the p53 pathway and induce cell-cycle arrest mediated by p21, and apoptosis mediated by BAX [58,59]. Cancer cells over-expressing wild-type p53 are deficient to the cytotoxic effect of paclitaxel [60]. These reports indicate that the p53 pathway is related to the cytotoxic mechanism of spindle poison, although the details of this process are not clear.

The four anti-metabolites tested did not show significant luciferase activity. They were reported not to be mutagenic in the Ames tests, but to be genotoxic in some genotoxic tests using mammalian cells, as indicated in Table. Methotrexate and 6-mercaptopurine were negative in the *in vivo* carcinogenicity tests in rodents. 5-Fluorouracil was described as equivocal in Table 1, because it is carcinogenic in mice but may not to be carcinogenic in rats and humans [61]. 5-Fluorouracil, a widely used anti-cancer agent is known to induce transactivation of p53 and p21 and to cause G1/S arrest in MCF-7 cells [62,63]. 5-Fluorouracil activates p53 via non-DNA-damaging stress, which induced by perturbations of nucleotide pools [64]. The activation of the stress-response pathway of p53 was regulated by post-translational modification, and the modification patterns differed with the type of stress pathways [65]. It is thought that the phosphorylation status of p53 or the activated pathway of p53, induced by anti-metabolites was different from the p53 activation mechanisms of this assay, and, therefore, anti-metabolites failed to induce significant luciferase activity in this assay. Hydroxyurea, DNA replication inhibitor and ribonucleotide reductase inhibitor, showed low levels of H2AX phosphorylation induced by accumulation by DSBs because of replication fork collapse [66]. This data suggest that this assay system does not only detect DSBs and does not simply detect transcription activity of p53.

As shown in Table 1, all genotoxicity tests agree well with the results of Ames-positive chemicals, but the concordance with Ames-negative chemicals is very low in commonly used mammalian genotoxicity tests, except for our assay. In particular, the anti-metabolites, except 5-fluorouracil, were positive in the commonly used *in vitro* mammalian genotoxicity tests, but not in our assay, although they were negative *in vivo* carcinogenicity in rodents. This false-positive result is important issue in the prediction of carcinogenicity *in vivo*. Our genotoxicity tests showed better concordance with carcinogenicity in rodents in this set.

The high rate of false positive result in vitro mammalian genotoxicity tests, such as chromosome aberration tests and mouse lymphoma test is critical problem. p53 is known to response to non-genotoxic stress such as hypoxia, pH change, and hyperosmotic stress. These factors may be potential cause of false positive result in this assay. In fact, this assay system was affected by osmotic pressures attained by addition of sodium chloride over 5 mg/mL (data not shown). The compounds, which inhibit protein synthesis, or, which directly affect promoter region of reporter plasmid, may bring inadequate results. In addition, the high concentrations of test chemicals and the cytotoxicity discussed as further potential sources of false positive in vitro mammalian genotoxicity assay [67]. To reduce inadequate results, this assay system should be carried out in appropriate conditions.

Recently, several genotoxicity tests, based on expression of p53 itself or on p53-target genes using human cells, have been developed [68–71]. Each test could detect various genotoxic agents, although the response profiles differed between tests, for example, anti-metabolites were positive in some test systems, because different p53 target genes were used. Among these reported tests, our genotoxicity test produced the highest intensity exposure to the genotoxic agents that induce double strand breaks.

To predict human genotoxicity it is very important to conduct tests using human cells. DNA repair mechanisms in human are different from those in rodents. UV-DDB (XPE) is deficient in rodent cells due to decreased expression of DDB2, so they are defective in p53-controlled GGR [72]. Rodents or other systems, in which the p53 role in GGR is compromised, are of limited applicability as surrogates for humans risk assessment.

Taken together, our genotoxicity test can detect various classes of genotoxic agents; topoisomerase inhibitors, intercalaters, alky-

lating agents, DNA cross-linkers, bulky adducts producers, and spindle poisons, though our test system cannot detect the genotoxic effect of anti-metabolite or HDAC inhibitors. In particular, this assay was highly sensitive to the genotoxic agents that induce DSBs, which correlate with initiation of genome instability. Furthermore, this assay offers several advantages, as follows; use of human cells, use of microplates and rapidity.

In conclusion, it is suggested that this genotoxicity test system, based on p53R2 gene expression, using human cell lines, can become a valuable tools for screening potential human genotoxins and for predicting human carcinogenicity.

Conflict of interest

None.

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