analysis of acetaminophen toxicity in mouse liver. Toxicol. Sci., **65**, 135-150.

Snedecor, G.W. and Cochran, W.G. (1989): Statistical Methods, 8th ed., Iowa State University Press.

Tarloff, J.B., Khairallah, E.A., Cohen, S.D. and Glodstein, R.S. (1996): Sex- and age-dependent acetaminophen hepato- and nephrotoxicity in Sprague-dawley rats: Role of tissue accumulation, nonprotein sulphydryl depletion, and covalent binding. Fundam. Appl. Toxicol., **30**, 13-22.

Urushidani, T. and Nagao, T. (2005): Toxicogenomics: The Japanese initiative. In (Borlak, J., ed.), Handbook of Toxicogenomics - Strategies and Applications. Wiley-VCH, pp.623-631.

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Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells

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Abstract

The recent finding that acrylamide (AA), a potent carcinogen, is formed in foods during cooking raises human health concerns. In the present study, we investigated the genotoxicity of AA and its metabolite glycidamide (GA) in human lymphoblastoid TK6 cells examining three endpoints: DNA damage (comet assay), clastogenesis (micronucleus test) and gene mutation (thymidine kinase (*TK*) assay). In a 4 h treatment without metabolic activation, AA was mildly genotoxic in the micronucleus and *TK* assays at high concentrations (>10 mM), whereas GA was significantly and concentration-dependently genotoxic at all endpoints at ≥0.5 mM. Molecular analysis of the *TK* mutants revealed that AA predominantly induced loss of heterozygosity (LOH) mutation like spontaneous one while GA-induced primarily point mutations. These results indicate that the genotoxic characteristics of AA and GA were distinctly different: AA was clastogenic and GA was mutagenic. The cytotoxicity and genotoxicity of AA were not enhanced by metabolic activation (rat liver S9), implying that the rat liver S9 did not activate AA. We discuss the in vitro and in vivo genotoxicity of AA and GA.

Keywords: Acrylamide; Gycidamide; Genotoxicity; *TK* mutation; Metabolic activation

1. Introduction

Acrylamide (AA) is a synthetic chemical that has been produced since the early 1950s. Because AA polymerizes easily to an adhesive gel, it has been widely used in industry for water flocculation, soil coagulation and grouts. Because it had been believed that humans are rarely exposed to AA under ordinary circumstances, concern was centered only on occupational exposure [1]. In 2000, however, Tareke et al. [2] reported that AA was unexpectedly discovered in cooking foods. It forms during frying and baking principally by a Maillard reaction between asparagine residues and glucose [3,4]. This finding raises concerns about the health risks of AA for the general population [5].

According to toxicological studies, AA is neurotoxic for animals and human [6,7], and the International

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Agency for Research on Cancer classifies it as 2A, a probable human carcinogen [1]. AA is also genotoxic in somatic and germinal cells in in vitro and in vivo [8]. In vivo examination [8] AA is metabolized to the epoxide derivative glycidamide (GA), presumably by cytochrome P4502E1 (CYP2E1) [9]. GA may be more toxic than AA because it reacts quickly with DNA and other biological macromolecules, and it is positive in most genotoxicity tests [8]. AA, on the other hand, is inactive in bacterial and some in vitro mammalian gene mutation assays, but it induces sister chromatid exchanges and chromosome aberrations in vitro and in vivo [8]. AA may have indirect genotoxic mechanisms, such as protein binding, spindle disturbance or hormonal imbalance, which could lead to tumors [10,11]. Thus, the genotoxic mechanism of AA is unclear.

In the present study, we used human lymphoblatoid TK6 cells to investigate the genotoxicity of AA and GA and its mechanisms. TK6 cells are widely used for the thymidine kinase (*TK*) gene mutation assay and can also be used in the in vitro micronucleus (MN) and comet (COM) assays. The *TK* gene mutation assay detects a wide range of genetic damage, including gene mutations, large-scale chromosomal deletions, recombination and aneuploidy [12], while other mammalian gene mutation assays, such as the *HPRT* and transgenic *LacZ* and *LacI* gene assays, detect only point mutations and small deletions [13]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumably relevant to carcinogenesis. Molecular analysis of the TK mutants induced by AA or GA can help elucidate their genotoxic mechanisms. In addition, because it uses a human cell line, the *TK* assay is appropriate for human hazard evaluation.

2. Materials and methods

2.1. Cell culture, chemicals and treatment

The TK6 human lymphoblastoid cell line has been described previously [14]. The cells were grown in RPMI1640 medium (Gibco-BRL, Life technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 10^5 to 10^6 cells/ml at 37 °C in a 5% CO_2 atmosphere with 100% humidity.

AA (CAS # 79-06-1) and GA (CAS # 5694-00-8) were purchased from Wako Pure Chemical Co. (Tokyo). We dissolved them in phosphate-buffered saline just before use. *N*-di-*N*-butylnitrosamine (DBN) (CAS # 924-16-3) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and dissolved in DMSO for use. Post-mitochondrial supernatant fractions of liver homogenate (S9) were purchased from Kikkoman Co. Ltd. (Noda, Chiba, Japan), which were prepared from the liver of phenobarbital- and 5,6-benzoflavone-treated SD rats. We prepared a 10 ml S9 mix with 4 ml S9 fraction and 2 ml each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP and 150 mM KCl.

We treated 20 ml aliquots of cell suspensions (5.0×10^5 cells/ml) at 37 °C for 4 h with serially diluted AA or GA, washed them once, re-suspended them in fresh medium, and cultured them in new flasks for the MN and *TK* assays or diluted and plated them for survival measurement (PE0). We treated the cultures with AA both in the absence and presence of 5% S9 mix.

2.2. Comet assay

After treating the cells for 4 h with AA or GA, we prepared slides for alkaline COM assay as previously reported [15]. Briefly, the cells were suspended in 0.5% agarose-LGT (Nakalai Tesque Inc., Kyoto, Japan), quickly layered on a slide (Matsunami Glass Ind. Ltd., Osaka, Japan) coated with 1% agarose GP-42 (Nakalai Tesque Inc.), and covered with 0.5% agarose-LGT. We immersed the slide in alkaline lysing solution (pH 13) for 1 h, electrophoresed it for 15 min after the unwinding treatment, fixed the cells with 70% ethanol, and stained them with SYBER green (Molecular Probes, Eugene, OR) according to the manufacturer's recommendation. We observed the cells by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and the tail length of the comet image was measured. We statistically analyzed the difference between the non-treated and treated plates with the Dunnett's test after one-way ANOVA [16].

2.3. Micronuclei test

Forty-eight hours after treatment, we prepared the MN test samples as previously reported [17]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold glacial acetic acid in methanol (1:3), and resuspended in methanol containing 1% acetic acid. We placed a drop of the suspension on a clean glass slide and allowed it to air-dry. We stained the cells with 40 μg/ml acridine orange solution and immediately observed them by Olympus model BX50 fluorescence microscope. At least, 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test. The concentration–response relationship was evaluated by the Cochran–Armitage trend test [18].

2.4. TK gene mutation assay

The TK6 cell cultures were maintained for 3 days after treatment to permit expression of the *TK* deficient phenotype. To isolate the *TK* deficient mutants, we seeded cells from each

culture into 96-microwell plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluorothymidine (TFT). We also plated them at 1.6 cells/well in the absence of TFT for the determination of plating efficiency (PE3). All plates were incubated at 37 °C in 5% CO_2 in a humidified incubator. The TK assay produces two distinct phenotypic classes of *TK* mutants: normally growing (NG) mutants had the same doubling time (13–17 h) as the wild type cells, and slowly growing (SG) mutants had a doubling time of >21 h. The difference is thought to be due to a putative gene near the *TK* gene. NG mutants result mainly from intragenic mutations, such as point mutations and small deletions, while SG mutants result from gross genetic changes extending beyond the *TK* gene [19]. We scored for the colonies in the PE plates and for the colonies for normal-growing *TK* mutants in the TFT plates at 14th day after plating. We then refed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing *TK* mutants. Mutation frequencies were calculated according to the Poisson distribution [20]. The data were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [21].

2.5. Molecular analysis of TK mutants

Genomic DNA was extracted from *TK* mutant cells and used as a template for the polymerase chain reaction (PCR). We analyzed for loss of heterozygosity (LOH) at the human *TK* gene by PCR products as described previously [22]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that contains frameshift mutations. Another primer set for amplifying parts of the β-globin were also prepared. We used quantitative-multiple PCR to co-amplify the three regions and to identify and quantify the PCR products. We analyzed them with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and classified the mutants into "none LOH", "hemizygous LOH" or "homozygous LOH". To determine the extent of LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis described previously [22]. The results were processed by GenoTyper™ software (PE Biosystems) according to the manufacturer's guidelines.

3. Results

3.1. Cytotoxic and genotoxic responses to AA and GA

Fig. 1a shows the effect of AA on relative survival (RS), mutation frequency (*TK* assay) and number of micronucleated cells per 1000 cells examined. AA was concentration-dependently cytotoxic, permitting about 20% RS at the maximum concentration (14 mM), while its genotoxicity and clastogenicity were weak. We repeated the experiment because of the weak genotoxicity. AA showed negative in the first *TK* assay, but positive in the second statistically. In MN test, both experiments showed statistically positive. GA, in contrast, was significantly genotoxic even at concentrations that were not severely cytotoxic (Fig. 1b). At the maximum concentration (2.4 mM), GA induced *TK* mutation frequencies that were about 20 times and MN fre-

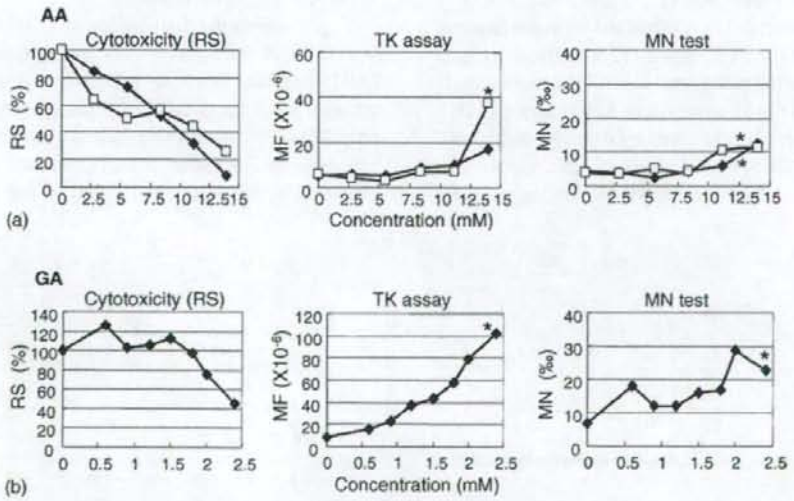

Fig. 1. Cytotoxic (relative survival, RS), genotoxic responses (TK assay and MN test) of TK6 cells treated with AA (a) or GA (b) for 4 h without metabolic activation. The AA experiment was repeated to confirm the result because of the weak genotoxicity. Closed and open symbols are first and second experiment, respectively. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P<0.05$).

Table 1
Cytotoxic and mutational responses to AA and GA, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) TK-mutants

Treatment	Cytotoxic and mutational response			LOH analysis at TK gene			
	RS (%)	MF ($\times 10^{-6}$)	% SG	No.	None LOH	Hemi-LOH	Homo-LOH
Vehicle [16]	100	2.19	56	56			
NG mutants				19	14(74)	3(16)	2(11)
SG mutants				37	0(0)	9(24)	28(76)
AA (14 mM, 4 h)	40	18.9	54	48			
NG mutants				22	11(50)	11(50)	0(0)
SG mutants				26	0(0)	13(50)	13(50)
GA (2.2 mM, 4 h)	12	55.5	36	44			
NG mutants				28	26(93)	2(7)	0(0)
SG mutants				16	0(0)	6(38)	10(62)

quencies at about four times the spontaneous level. We detected two distinct phenotypic classes of *TK* mutants in *TK* assay: NG and SG mutants. AA did not affect the proportion of SG mutants, while GA treatment lowered it (Table 1). This implies that GA induced primarily point mutations. In the COM assay, even at the highest concentration, AA did not induce DNA damage, while GA did so strongly starting at 0.6 mM (Fig. 2).

3.2. Molecular analysis of TK mutants

The *TK* mutants were independently isolated from the cells treated with 14 mM AA or 2.2 mM GA for 4 h. Table 1 shows the cytotoxicity (RS) and *TK* mutation frequency (MF) and proportion of SG mutants (% SG) by the treatment. Genomic DNA extracted from the mutants was subjected by the PCR-based LOH analysis to classify the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH) and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [13]. We analyzed 48 AA-induced and 44 GA-induced *TK* mutants and compared them to those of spontaneously occurring *TK* mutants described previously [16]. The fraction of hemi-LOH in AA-induced mutants, in which 50% each of NG and SG mutants exhibited hemi-LOH, was higher than in spontaneous mutants, indicating that AA-induced primarily deletions. GA, on the other hand, induced primarily NG mutants, and most (93%) of them were the non-LOH type, which is presumably generated by point and other small intragenic mutations. Among 16 GA-induced SG mutants, the percentages that were hemi-LOH (38%) and homo-LOH (62%) were similar to those observed in spontaneous SG mutants. Fig. 3 shows the mutation spectra of *TK* mutants found among treated and untreated TK6 cells. GA and ethyl methane sulfonate, an alkylating agent, produce similar spectra, as do AA and X-radiation.

Fig. 4 shows the distribution of LOH in AA-induced ($n=37$), GA-induced ($n=17$) and spontaneous ($n=29$) LOH mutants. Because the majority of GA-induced mutants were the non-LOH type, we were able to map only 17 GA-induced LOH mutants. As a particular characteristic of AA-induced LOH mutants, we frequently observed small deletions limited to the *TK* locus. The

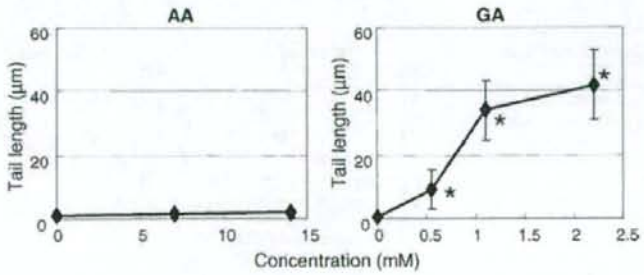

Fig. 2. COM assay results in TK6 cells treated with AA or GA for 4 h without metabolic activation. Asterisk (*) statistically significant in the Dunnett's tests ($P<0.05$).

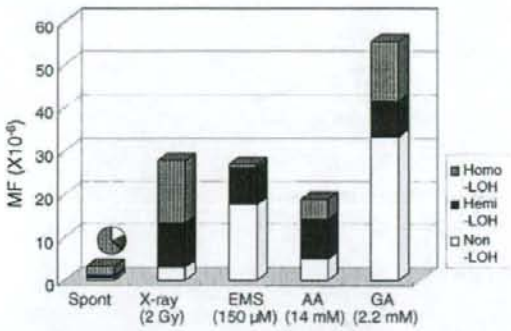

Fig. 3. Frequency and spectra of TK mutations in spontaneous and X-ray-induced (2 Gy), EMS-induced (150 μM, 4 h), AA-induced (14 mM, 4 h) and GA-induced (2.2 mM, 4 h) TK mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of normally growing (NG) and slowly growing (SG) mutants and the results of molecular analysis (Table 1). The data of spontaneous, X-ray-induced and EMS-induced mutation spectra were taken from our previous paper [13].

distribution of LOH in GA-induced and spontaneous LOH mutants was similar.

3.3. Cytotoxicity and genotoxicity of AA under metabolic activation

Rat liver S9 mix did not influence the cytotoxicity or genotoxicity of AA but it did enhance the activity of DBN, the positive control chemical (Fig. 5).

4. Discussion

A large number of studies about the in vitro genotoxicity of AA have been reported [8]. AA has consistently been negative in bacterial gene mutation assay in both the presence and absence of metabolic activation [23–25] but positive in chromosome aberration and sister chromatid exchange tests in Chinese hamster cell lines [24–26]. In mammalian cell assays, AA induces *Tk* but not *Hprt* gene mutations [24,25,27,28], and is negative in the COM assay even at high concentrations [27]. These results suggest that AA is clastogenic without directly damaging DNA. GA, on the other hand, is positive in most in vitro genotoxicity tests and is recognized as a mutagen [8,27,29]. In the present study, the higher concentrations of AA were positive in the MN and TK assay but negative in the comet assay. According to the in vitro genotoxicity test guideline, however, AA may be negative [30], because the guideline suggests that the maximum concentration should be 10 mM. Because the genotoxic responses at higher concentrations were reproducible, AA may be genotoxic, but its effect is very weak. GA, in contrast, was positive in all the assays, even under conditions of low cytotoxicity. These results are consistent with the reports described above.

The mammalian *TK* gene mutation assay can detect a wide range of genetic changes, including point mutations, small deletions, large-scale chromosomal deletions, inter-allelic recombination and aneuploidy, while

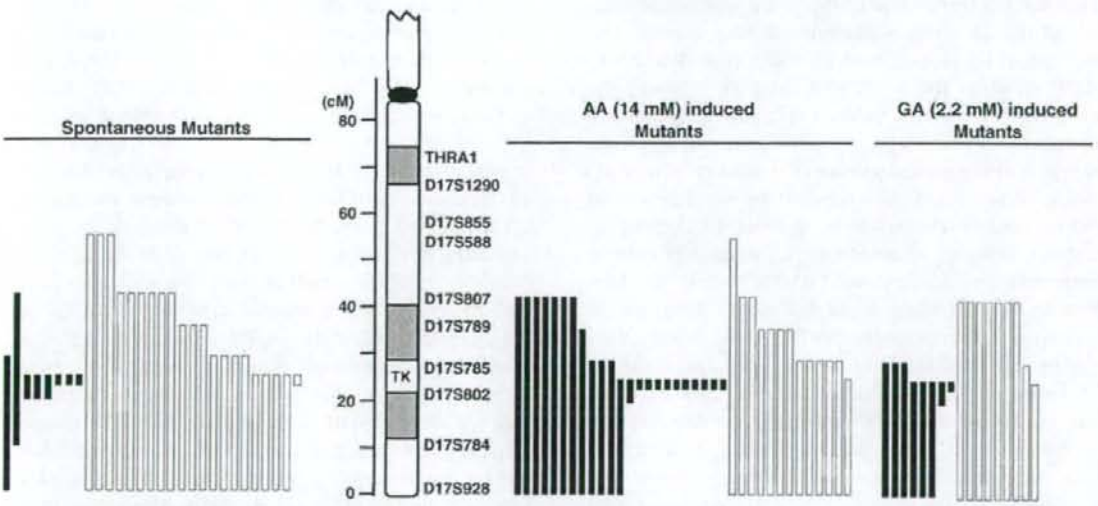

Fig. 4. The extent of LOH in spontaneous, AA-induced and GA-induced LOH mutants from TK6 cells. We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human TK locus maps to 17q23.2. Open and closed bars represent homo-LOH and hemi-LOH, respectively. The length of the bar indicates the extent of the LOH. We analyzed 29 spontaneous mutants (10 NG and 19 SG mutants), 37 AA-induced mutants (11 NG and 26 SG) and 17 GA-induced mutants (2 NG and 15 SG). The data on spontaneous mutants were taken from our previous paper [13].

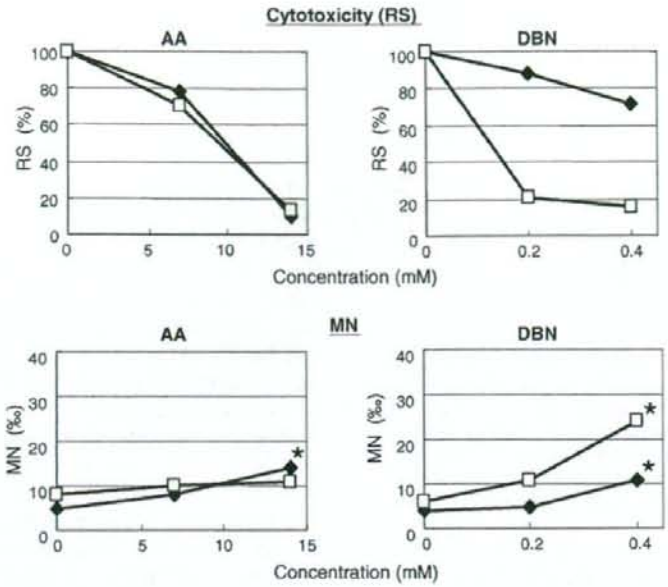

Fig. 5. Cytotoxicity (RS) and MN induction in TK6 cells treated with AA or DBN for 4 h in the presence (open symbol) or absence (closed symbol) of rat liver S9. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

the bacterial and mammalian *HPRT* gene mutation assays detect only point mutations and small intragenic deletions [13]. AA was positive only in the *TK* mutation assay, suggesting that AA causes predominantly large-scale chromosomal changes. Our molecular analysis of the *TK* mutants supported this hypothesis. The majority of the AA-induced *TK* mutants showed hemi-LOH, which is the result of a deletion, although the other types were also induced (Fig. 3). Deletions are thought to result from the repair of double strand breaks by non-homlogous end-joining [13]. Radiation-induced double strand breaks are repaired by non-homlogous end-joining, which leads to hemi-LOH. LOH-mapping analysis, however, revealed that AA frequently induces intermediate-sized deletions (100–3000 kb); the deletions encompass exons 4 and 7 of the *TK* locus but do not extend to the microsatellites loci of the vicinity. This type of deletion is rarely observed in radiation-inducing *TK* mutants [13]. Because the COM assay indicated that AA did not induce DNA damage, the deletion may not be caused by DNA damage directly. Mechanisms associated with global genomic instability should also be considered [10] because the LOH patterns, except for the intermediate-sized deletions, are generally similar to those observed in spontaneous mutants. Most GA-induced *TK* mutants, on the other hand, were the non-LOH type, as were most spontaneous ones, strongly supporting the positive results in bacterial gene mutation assay [29]. In contrast to AA, GA is a mutagen, inducing primarily point mutations.

AA is known to be metabolized to GA by CYP2E1 [9]. GA, an epoxide, forms adducts directly with DNA and protein, causing cytotoxicity and genotoxicity. GA forms mainly *N*7-(2-carbamoyl-2-hydroxyethyl) guanine and *N*3-(2-carbamoyl-2-hydroxyethyl) adenine and reacts with hemoglobin and cytoskeletal proteins [31–33]. Rat S9, however, did not affect AA cytotoxicity or genotoxicity, although it did enhance the cytotoxicity and genotoxicity of DBN, which is also metabolized by CYP2E1. This suggests that rat S9 does not work for activating AA. AA and GA are detoxified through glutathione conjugation, and GA is also detoxified by epoxy hydrolase (EH), which catalyzes the hydrolysis of GA to dihidroxy propionamide [34,35]. Other in vitro studies also failed to demonstrate the enhancement of AA genotoxicity by rat S9 [36,37]. Our results do not mean that AA is always detoxified rather than activated because DNA adducts are found in mice and rats given oral AA, and the genotoxicity of AA is consistently observed in in vivo studies [8,31,36,37]. Recently, Manjanatha et al. demonstrated in transgenic Big Blue™ mice that AA as well as GA induces endogenous *Hprt* and transgenic *cII* mutation at same level, and both chemicals cause predominantly base substitutions and frameshift mutations.

This result may indicate that AA is metabolized to GA in vivo [38]. Tests that use rat liver S9 for metabolic activation may not be appropriate for in vitro investigations of AA genotoxicity and metabolism. Transgenic cells expressing CYP2E1, however, would be useful for demonstrating the in vitro genotoxicity of AA [39].

In conclusion, AA is weakly genotoxic, causing chromosome aberrations and a type of genomic instability. GA, its epoxide metabolite, is highly reactive with DNA. GA is a strong mutagen, inducing predominantly point mutations, and it may contribute to human cancers.

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References

[1] IARC, Acrylamide, IARC Monographs on the Evaluation of Carcinogen Risk to Human: Some Industrial Chemicals, vol. 60, International Agency for Research on Cancer, Lyon, 1994, pp. 389–433.
[2] E. Tareke, P. Rydberg, P. Karlsson, S. Eriksson, M. Tornqvist, Acrylamide: a cooking carcinogen? Chem. Res. Toxicol. 13 (2000) 517–522.
[3] R.H. Stadler, I. Blank, N. Varga, F. Robert, J. Hau, P.A. Guy, M.C. Robert, S. Riediker, Acrylamide from Maillard reaction products, Nature 419 (2002) 449–450.
[4] d.S. Mottram, B.L. Wedzicha, A.T. Dodson, Acrylamide is formed in the Maillard reaction, Nature 419 (2002) 448–449.
[5] J.M. Rice, The carcinogenicity of acrylamide, Mutat. Res. 580 (2005) 3–20.
[6] E.J. Lehning, C.D. Balaban, J.F. Ross, M.A. Reid, R.M. LoPachin, Acrylamide neuropathy. I. Spatiotemporal characteristics of nerve cell damage in rat cerebellum, Neurotoxicology 23 (2002) 397–414.
[7] R.M. LoPachin, C.D. Balaban, J.F. Ross, Acrylamide axonopathy revisited, Toxicol. Appl. Pharmacol. 188 (2003) 135–153.
[8] K.L. Dearfield, G.R. Douglas, U.H. Ehling, M.M. Moore, G.A. Sega, D.J. Brusick, Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk, Mutat. Res. 330 (1995) 71–99.
[9] S.C. Sumner, T.R. Fennell, T.A. Moore, B. Chanas, F. Gonzalez, B.I. Ghanayem, Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice, Chem. Res. Toxicol. 12 (1999) 1110–1116.
[10] M. Friedman, Chemistry, biochemistry, and safety of acrylamide. A review, J. Agric. Food Chem. 51 (2003) 4504–4526.
[11] H.M. Bolt, Genotoxicity–threshold or not? Introduction of cases of industrial chemicals, Toxicol. Lett. 140–141 (2003) 43–51.
[12] H.L. Liber, W.G. Thilly, Mutation assay at the thymidine kinase locus in diploid human lymphoblasts, Mutat. Res. 94 (1982) 467–485.
[13] M. Honma, Generation of loss of heterozygosity and its dependency on p53 status in human lymphoblastoid cells, Environ. Mol. Mutagen. 45 (2005) 162–176.
[14] M. Honma, M. Hayashi, T. Sofuni, Cytotoxic and mutagenic responses to X-rays and chemical mutagens in normal and p53-mutated human lymphoblastoid cells, Mutat. Res. 374 (1997) 89–98.
[15] K. Sekihashi, H. Saitoh, Y. Sasaki, Genotoxicity studies of stevia extract and steviol by the comet assay, J. Toxicol. Sci. 27 (Suppl. 1) (2002) 1–8.
[16] M. Watanabe-Akanuma, T. Ohta, Y.F. Sasaki, A novel aspect of thiabendasole as a photomutagen in bacteria and cultured human cells, Mutat. Res. 158 (2005) 213–219.
[17] L. Zhan, H. Sakamoto, M. Sakuraba, D.S. Wu, L.S. Zhang, T. Suzuki, M. Hayashi, M. Honma, Genotoxicity of microcystin-LR in human lymphoblastoid TK6 cells, Mutat. Res. 557 (2004) 1–6.
[18] T. Matsushima, M. Hayashi, A. Matsuoka, M. Ishidate Jr., K.F. Miura, H. Shimizu, Y. Suzuki, K. Morimoto, H. Ogura, K. Mure, K. Koshi, T. Sofuni, Validation study of the in vitro micronuclei test in a Chinese hamuster lung cell line (CHL/IU), Mutagenesis 14 (1999) 569–580.
[19] S.A. Amundson, H.L. Liber, A comparison of induced mutation at homologous alleles of the tk locus in human cells, Mutat. Res. 247 (1991) 19–27.
[20] E.E. Furth, W.G. Thilly, B.W. Penman, H.L. Liber, W.M. Rand, Quantitative assay for mutation in diploid human lymphoblasts using microtiter plates, Anal. Biochem. 110 (1981) 1–8.
[21] T. Omori, M. Honma, M. Hayashi, Y. Honda, I. Yoshimura, A new statistical method for evaluating of L5178Ytk± mammalian cell data using microwell method, Mutat. Res. 517 (2002) 199–208.
[22] M. Honma, M. Momose, H. Tanabe, H. Sakamoto, Y. Yu, J.B. Little, T. Sofuni, M. Hayashi, Requirement of wild-type p53 protein for maintenance of chromosomal integrity, Mol. Carcinog. 28 (2000) 203–214.
[23] E. Zeiger, B. Anderson, S. Haworth, T. Lawlor, K. Mortelmans, W. Speck, Salmonella mutagenicity tests: III. Results from the testing of 255 chemicals, Environ. Mutagen. 9 (Suppl. 9) (1987) 1–109.
[24] H. Tsuda, C.S. Shimizu, M.K. Taketomi, M.M. Hasegawa, A. Hamada, K.M. Kawata, N. Inui, Acrylamide; induction of DNA damage, chromosomal aberrations and cell transformation without gene mutations, Mutagenesis 8 (1993) 23–29.
[25] A.G. Knaap, P.G. Kramers, C.E. Voogd, W.G. Bergkamp, M.G. Groot, P.G. Langebroek, H.C. Mout, J.J. van der Stel, H.W. Verharen, Mutagenic activity of acrylamide in eukaryotic systems but not in bacteria, Mutagenesis 3 (1988) 263–268.
[26] T. Sofuni, M. Hayashi, A. Matsuoka, M. Sawada, Mutagenicity tests on organic chemical concominants in city water and related compounds. II. Chromosome aberration tests in cultured mammalian cells, Eisei Shiken. Hok. 103 (1985) 64–75.
[27] M. Baum, E. Fauth, S. Fritzen, A. Herrmann, P. Mertes, K. Merz, M. Rudolphi, H. Zankl, G. Eisenbrand, Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood, Mutat. Res. 580 (2005) 61–69.
[28] M.M. Moore, A. Amtower, C. Doerr, K.H. Brock, K.L. Dearfield, Mutagenicity and clastogenicity of acrylamide in L5178Y mouse lymphoma cells, Environ. Mutagen. 9 (1987) 261–267.
[29] K. Hashimoto, H. Tanii, Mutagenicity of acrylamide and its analogues in *Salmonella typhimurium*, Mutat. Res. 158 (1985) 129–133.
[30] C.S. Aaron, G. Bolcsfoldi, H.R. Glatt, M. Moore, Y. Nishi, L. Stankowski, Theiss F J., E. Thompson, Mammalian cell gene mutation assays working group report, Mutat Res. 312 (1994) 235–239.

[31] d.C. Gamboa, M.I. Churchwell, L.P. Hamilton, L.S. Von Tungeln, F.A. Beland, M.M. Marques, D.R. Doerge, DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice, Chem. Res. Toxicol. 16 (2003) 1328–1337.

[32] D.M. Lapadula, M. Bowe, C.D. Carrington, L. Dulak, M. Friedman, M.B. Abou-Donia, In vitro binding of [14C]acrylamide to neurofilament and microtubule proteins of rats, Brain Res. 481 (1989) 157–161.

[33] S.C. Sumner, C.C. Williams, R.W. Snyder, W.L. Krol, B. Asgharian, T.R. Fennell, Acrylamide: a comparison of metabolism and hemoglobin adducts in rodents following dermal, intraperitoneal, oral, or inhalation exposure, Toxicol. Sci. 75 (2003) 260–270.

[34] B. Paulsson, A. Rannug, A.P. Henderson, B.T. Golding, M. Tornqvist, M. Warholm, In vitro studies of the influence of glutathione transferases and epoxide hydrolase on the detoxification of acrylamide and glycidamide in blood, Mutat. Res. 580 (2005) 53–59.

[35] S.C. Sumner, L. Selvaraj, S.K. Nauhaus, T.R. Fennell, Urinary metabolites from F344 rats and B6C3F1 mice coadministered acrylamide and acrylonitrile for 1 or 5 days, Chem. Res. Toxicol. 10 (1997) 1152–1160.

[36] A. Besaratinia, G.P. Pfeifer, Genotoxicity of acrylamide and glycidamide, J. Natl. Cancer Inst. 96 (2004) 1023–1029.

[37] A. Besaratinia, G.P. Pfeifer, DNA adduction and mutagenic properties of acrylamide, Mutat. Res. 580 (2005) 31–40.

[38] M.G. Manjanatha, A. Aidoo, S.D. Shelton, M.E. Bishop, L.P. MacDaniel, D.R. Doerge, Genotoxicity of acrylamide and its metabolite glycidamide administrated in drinking water to male and female Big Blue mice, Environ. Mol. Mutagen., in press (Epub ahead of prints).

[39] H. Glatt, H. Schneider, Y. Liu, V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals, Mutat. Res. 580 (2005) 41–52.

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Relationship between p53 status and 5-fluorouracil sensitivity in 3 cell lines

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Abstract

Mouse lymphoma L5178Ytk$^{+/-}$ (MOLY) cells and human lymphoblastoid TK6 and WTK-1 cells are widely used to detect mutagens in vitro. MOLY and WTK-1 cells have a p53 mutation, while TK6 cells, which were derived from the same parental line as WTK-1 cells, do not. In this study, we tested the clastogen 5-fluorouracil (5-FU) in the Tk assay and the in vitro micronucleus (MN) assay in MOLY, TK6, and WTK-1 cells to clarify whether differential responses were related to p53 gene status. We also determined the effect of 5-FU on the frequency of apoptotic cells and on cell cycle distribution in each cell line. Furthermore, we measured the activity of the 5-FU metabolizing enzymes (thymidylate synthetase (TS), dihydrouracil dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), and thymidine phosphorylase (TP)) in each cell line.

We treated MOLY cells with 1.0–8.0 μg/mL 5-FU for 3 h and TK6 and WTK-1 cells with 1.56–25 and 3.13–50 μg/mL, respectively, for 4 h. In MOLY cells, the mutation frequency (MF) and MN frequency increased. In WTK-1 cells, the MN frequency but not the MF increased. In TK6 cells, neither the MF nor the MN frequency increased. Furthermore, the IC_{50} of 5-FU was lower in MOLY cells than in the human cells. The response to 5-FU treatment differed in other ways as well. At the same level of cytotoxicity, the frequency of apoptotic cell was highest in TK6 cells. The cell cycle was delayed just after treatment in MOLY cells while the delay appeared 24 h later in TK6 and WTK-1 cells. Nothing in our analysis, however, revealed marked differences between the cell lines that could account for the severe cytotoxic and mutagenic responses that 5-FU elicited only in MOLY cells.

5-FU is phosphorylated by OPRT and TP and detoxified by DPD. MOLY cells have higher OPRT activity and markedly lower DPD and TP activity than TK6 and WTK-1 cells. The content of TS, however, the target enzyme of 5-FU, was similar in all cell lines, suggesting that 5-FU was more readily phosphorylated and less readily detoxified in MOLY cells than in TK6 and WTK-1 cells.

MOLY cells were more sensitive to 5-FU than WTK-1 cells even though both have a mutated p53 gene, suggesting that the different responses to 5-FU were due to differences in 5-FU metabolism rather than the p53 status.

Keywords: 5-fluorouracil; p53; *Tk* assay; In vitro micronucleus assay

1. Introduction

Mouse lymphoma L5178Ytk$^{+/-}$ (MOLY) cells are widely used in *Tk* mutation assays [1–5]. MOLY cells are heterozygous at the *Tk* locus, where loss of heterozygos-

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ity (LOH) leads to the detection of mutants [6]. Human lymphoblastoid TK6 and WTK-1 cells are also heterozygous at the *Tk* locus and are also widely used in the *Tk* mutation assay [7–10].

TK6 and WTK-1 cells are both derived from WIL-2 cells [11], but TK6 cells have a wild type p53 tumor suppressor gene while WTK-1 cells have a mutant p53 gene [12].

p53 is involved in DNA repair, the G_1 check point, and the induction of apoptosis [13]. Wild type p53 leads cells with repairable DNA damage into cell cycle arrest, allowing repair of the damage, and cells with severe DNA damage to apoptosis, although mutated p53 does not. Earlier studies have shown that, compared with p53 mutant or null cells, p53 wild type cells undergo apoptosis earlier or at a higher frequency following severe cytotoxicity induced by X-rays or certain chemicals [10,12,14–17]. p53 mutant cells differ from the wild type in their cytotoxic and mutagenic responses to some mutagens [3,18–20]. Some clastogens and spindle poisons are more cytotoxic in wild type cells than in mutant cells, and at equal levels of cytotoxicity, are more clastogenic in the mutant cells [10,21].

The spontaneous MF in WTK-1 cells is 100–200 $\times 10^{-6}$ while it is less than 10×10^{-6} in TK6 cells [3]. Furthermore WTK-1 cells are more resistant than TK6 cells to X-rays and other mutagens [3]. These differences may be due to differences in p53 status. Because TK6 and WTK-1 cells are genetically similar except for p53 status, they are useful for examining the function of p53 status.

p53 is mutated at codon 170 of exon 5 in MOLY cells and at codon 237 of exon 7 in WTK-1 cells [22]. Because both those codons are the part of the central DNA binding domain [13], it is likely that the mutant p53 proteins in MOLY and WTK-1 cells lose the same function. The spontaneous MF is about the same in MOLY [5] and WTK-1 cells, and MOLY cells are also more resistant to X-rays than p53 wild type cells [21]. Because no cell line with a wild type p53 gene has the same genomic background as MOLY cells, MOLY cells cannot be compared directly with a p53 wild type cell line. However the response of MOLY cells to mutagens is probably similar to that of WTK-1 cells, so MOLY cells would show the same genomic instability as WTK-1 cells (or other p53-mutated cells).

5-Fluorouracil (5-FU), a pyrimidine based analog, is used as an anticancer drug. When 5-FU is converted to 5-fluoro-2-deoxyuridylate monophosphate (FdUMP), it competitively inhibits DNA synthesis via inhibition of thymidylate synthetase (TS) [23–25]. 5-FU is metabolized by orotate phosphoribosyl transferase (OPRT) and thymidine phosphorylase (TP) and detoxified by dihydrouracil dehydrogenase (DPD) [24–27].

5-FU is clastogenic in mammalian cells [28] in vitro and in vivo [29], and is mutagenic to MOLY cells [5,30], but not to *Salmonella typhimurium* [28]. While 5-FU induces neither chromosomal aberrations nor sister chromatid exchanges in the human subjects studied so far [31], it is difficult to assess whether 5-FU is truly non-mutagenic in humans because all the human subjects have been patients. Furthermore, there are few reports on the mutagenicity or clastogenicity of 5-FU in human cell lines. Here we evaluated the mutagenicity and clastogenicity of 5-FU in human cell lines in an attempt to assess its risk to humans.

Because both WTK-1 and MOLY cells are p53 mutants, we were interested in determining whether both lines would respond similarly to 5-FU. We used both lines, and TK6 cells as well, to test the effects of 5-FU in the *Tk* and in vitro MN assays, and we also analyzed the effect of 5-FU on apoptosis and the cell cycle in each cell line. Furthermore we measured the activity of the 5-FU metabolizing enzymes (TS, DPD, OPRT, and TP) in each cell line.

2. Materials and methods

2.1. Chemicals

5-FU (CAS No. 51-21-8) was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan), and methyl methanesulfonate (MMS, CAS No. 66-27-3) from Aldrich Chemical Co. Inc. (Milwaukee, WI). 5-FU and MMS were dissolved in physiologic saline just before use. [6-^{14}C]–5-FU (1.85 GBq/mmol) and [6-^{3}H]–FdUMP (625 GBq/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA).

2.2. Cell lines and culture

MOLY, TK6, and WTK-1 cells were provided by Hatano Research Institute at the Food and Drug Safety Center. The cells were cultured in RPMI 1640 medium (Gibco, Invitrogen Corp., Carlsbad, CA) containing 10% (v/v) horse serum (JRH Biosciences, Lenexa, KS); 20% (v/v) horse serum was used to measure the plating efficiency and mutation frequency of MOLY cells. The cells were incubated at 37 °C in an atmosphere of 5% CO_2 and high humidity.

2.3. Selection of concentration

Considering its cytotoxicity, we tested 5-FU at 1, 2, 4, and 8 μg/mL in MOLY cells; 1.56, 3.13, 6.25, 12.5, and 25 μg/mL in TK6 cells; and 3.13, 6.25, 12.5, 25, and 50 μg/mL in WTK-1 cells. MMS was used as the positive control at 10 μg/mL in MOLY cells and 5 μg/mL in human cells.

2.4. Chemical treatment

In this study, MOLY cells doubled in 10.3 h, TK6 cells in 16.9 h, and WTK-1 cells in 19.9 h. Based on that, we treated MOLY cells for 3 h and the human cell lines for 4 h. We treated 1×10^7 cells (at 5×10^5/mL), with shaking, at 37 °C, washed them, adjusted them to a density of 2×10^5 cells/mL, and incubated them.

2.5. Tk assay

The *Tk* assay was performed by the microwell method [3,5]. A portion of the cells were seeded onto 96-well plates to measure the plating efficiency (PE) after 5-FU treatment (PE0). The remaining MOLY cells were incubated for 48 h and the human cells for 72 h to permit phenotypic expression. They were seeded onto 96-well plates in the presence of trifluorothymidine (TFT, CAS No. 70-00-8, Sigma Chemical Co., St. Louis, MO) for assessment of mutation frequency (MF). Another portion of the cells were seeded onto 96-well plates in the absence of TFT for the measurement of plating efficiency after the expression period (PE2 for MOLY cells, PE3 for TK6 and WTK-1 cells).

After incubation, the number of wells containing colonies were counted, and PE, MF, and relative survival (RS) from PE0 were calculated as previously described [5]. We also calculated the 5-FU concentration that caused 50% growth inhibition (IC_{50}) in RS0.

2.6. In vitro MN assay

We collected 10^6 to 3×10^6 cells 48 h after 5-FU treatment, and prepared the MN assay specimens as previously described [32]. The specimens stained with acridine orange (Wako) were observed with the aid of a fluorescence microscope with a 470–490 nm excitation filter. One thousand cells from each treatment group were observed and the number with MN was counted. To be judged a MN, the structure had to have a diameter that was less than half the diameter of the main nucleus. Cells having an abnormal main nucleus were excluded from this analysis.

2.7. Analysis of apoptosis and the cell cycle

For analysis of apoptosis and cell cycle distribution, we sampled 2×10^6 cells at 0.5, 24, and 48 h (all cell lines) or at 72 h (TK6 and WTK-1 cells) after 5-FU treatment. Apoptosis was analyzed by flow cytometry and nuclear morphology. For flow cytometry, MEBSTAIN Apoptosis Kit® (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan), which is based on the TUNEL method [33,34], was used. The number of TUNEL-positive cells per 10,000 cells was determined with a flow cytometer (FACScan, Becton-Dickinson, San Diego, CA) equipped with a 488 nm argon-ion laser at 15 mW with a 530/30 nm bandpass filter.

For nuclear morphology analysis, the cells were stained with Hoechst 33342 solution (Wako) and observed with the aid of a fluorescence microscope with a 330–385 nm excitation filter.

For analysis of cell cycle distribution, the cells were fixed with 70% (v/v) ethanol at −20 °C overnight, and stained with propidium iodide (Wako) for 30 min at room temperature. Ten thousand cells were analyzed on FACScan with a 585/42 nm bandpass filter. Mod Fit LT ver. 1.00 (Verity Software House Inc., Topsham, ME) was used for cell cycle analysis.

2.8. Measurement of enzyme activity

2.8.1. TS content

TS content was determined by quantifying [6-^{3}H]–FdUMP binding sites in the 105,000 g supernatant (cytosol) of cell homogenates based on the method described by Spears et al. [35], with minor modifications [36]. The samples were incubated with [6-^{3}H]–FdUMP in the presence of 5,10-methylene tetrahydrofolate for 20 min at 30 °C and radioactivity in the acid-insoluble fraction was measured with a liquid scintillation counter.

2.8.2. DPD, OPRT, and TP activity

DPD activity were measured as previously described, with minor modifications [35,37]. Briefly, cell line enzyme solutions were incubated with a reaction mixture containing 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.02 mM [6-^{14}C]–5-FU (56 nCi), and 100 mM NADPH at 37 °C for 10 or 30 min. The DPD activity was calculated as the ratio of the radioactivity of the catabolism fraction (dihydrofluorouracil and fluoro-β-alanine) to total radioactivity ([6-^{14}C]–5-FU plus the catabolism fraction).

OPRT activity was determined according to the modified method of Peters et al. [38] using [6-^{14}C]–5-FU (56 nCi) as the substrate. Cell line enzyme solutions were incubated with a reaction mixture containing 50 mM Tris–HCl buffer (pH 8.0), 10 mM NaF, 5 mM $MgCl_2$, 4 mM PRPP, and 0.02 mM [6-^{14}C]–5-FU (56 nCi) at 37 °C for 10 or 30 min. OPRT activity was calculated as the ratio of the radioactivity of the metabolism fraction (5-fluoro-2-deoxyuridylate monophosphate) to the total radioactivity ([6-^{14}C]–5-FU plus the metabolism fraction).

TP activity was measured as previously described [39]. Briefly, the cell line enzyme solution was incubated with a reaction mixture containing 50 mM Tris–HCl buffer (pH 8.0), 10 mM NaF, 5 mM $MgCl_2$, 4 mM deoxyribose-1-phosphate, 10 mM ATP, and 0.02 mM [6-^{14}C]–5-FU (56 nCi) at 37 °C for 10 or 30 min. TP activity was calculated as the ratio of the radioactivity of the metabolism fraction (5-fluorodeoxyuridine) to total radioactivity ([6-^{14}C]–5-FU plus the metabolism fraction).

2.9. Statistical analysis

We judged the *Tk* mutation assay as positive when treatment at least doubled the MF relative to the solvent control and linear regression analysis indicated concentration dependence. For the MN assay, the conditional binomial test was performed

in Microsoft Excel 2000. The χ^2-test was used to evaluate the statistical significance of the ratio of apoptotic to normal cells. We considered P-values <0.05 as significant. The Dunn test was used for multiple comparisons in the conditional binomial and χ^2-test. SAS, Windows edition (System release 7.10), was used for linear regression analysis and to calculate the IC_{50}.

3. Results

3.1. Cytotoxicity

5-FU was cytotoxic in all cell lines in a concentration-dependent manner (Fig. 1). In MOLY and TK6 cells, there were severe cytotoxicity in the highest dose. The IC_{50} of RS0 was 2.4 μg/mL in MOLY cells, 15.4 μg/mL in TK6 cells, and 30.6 μg/mL in WTK-1 cells.

3.2. Tk assay

5-FU increased MF in MOLY cells in a concentration-dependent manner ($P=0.021$, linear regression analysis), doubling the total MF relative to the solvent control at 2.0 μg/mL and quadrupling it at 4.0 μg/mL (Fig. 1). MF was not increased more than 2 times to the solvent control in TK6 and WTK-1 cells. Therefore 5-FU was not mutagenic to TK6 or WTK-1 cells while MMS, the positive control, was.

3.3. MN assay

5-FU increased the MN frequency to 5.5 times the control value at 2.0 μg/mL ($P=0.0003$) and 14.5 times the control value at 4.0 μg/mL ($P<0.0001$) in MOLY cells in a concentration-dependent manner (Fig. 2). In WTK-1 cells, 5-FU increased the MN frequency to 7.3 times the control value only at 50 μg/mL ($P=0.0001$). 5-FU was not clastogenic in TK6 cells while MMS, the positive control, was.

3.4. Measurement of apoptotic cells

5-FU at the higher concentrations significantly increased the ratio of cells in apoptosis 24 h after treatment in all cell lines ($P<0.0001$–0.0162, χ^2-test) (Fig. 3). TK6 cells showed the greatest response. At the same level of cytotoxicity, the levels of apoptosis in MOLY and WTK-1 cells were similar. We confirmed apoptosis by nuclear morphology analysis.

3.5. Cell cycle effects

Fig. 4 shows the time course of cell cycle distribution following treatment with various concentration of 5-FU. In MOLY cells, no G_2/M phase cells were evident 0.5 h after treatment and G_1 phase cells accumulated slightly. At 24 h, we observed cell cycle delay at 4.0 μg/mL

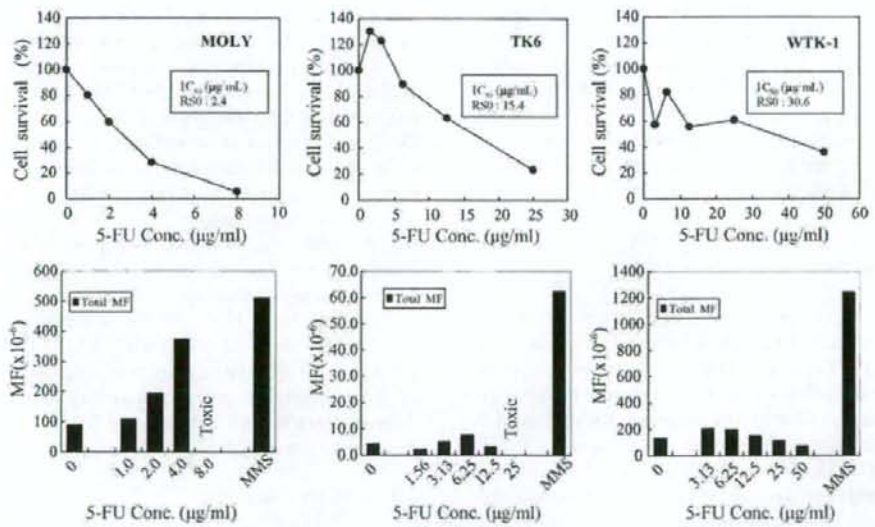

Fig. 1. The mutation frequency and cytotoxicity in tk assay after 5-FU treatment in MOLY, TK6 and WTK-1 cells. The percentage of cell survival (RS0, line graphs) and total MF (bar graphs) after 5-FU treatment (MOLY cells for 3 h and the human cell lines for 4 h) were shown. 5-FU was cytotoxic in all cell lines in a concentration-dependent manner. 5-FU increased MF in MOLY cells in a concentration-dependent manner, however not mutagenic to TK6 or WTK-1 cells.

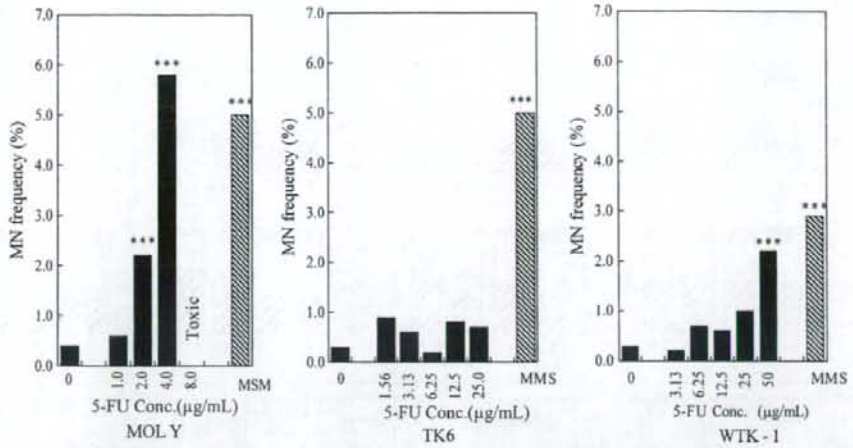

Fig. 2. The frequency of cells with micronuclei in MOLY, TK6 and WTK-1 cells after 5-FU treatment. The frequency of cells with MN (MN frequency) 48 h after 5-FU treatment was shown. One thousand cells from each treatment group were observed and the number with MN was counted. 5-FU significantly increased MN frequency in comparison with the control value at 2.0 ($P = 0.0003$) and 4.0 μg/mL ($P < 0.0001$) in MOLY cells. 5-FU increased MN frequency only at 50 μg/mL ($P = 0.0001$) in WTK-1 cells. 5-FU was not clastogenic in TK6 cells. ***$P < 0.001$, conditional binomial test.

5-FU. The cell cycle returned to normal 48 h after treatment.

In TK6 cells, we observed no cell cycle changes 0.5 h after treatment. G_1 phase cells accumulated in direct proportion to 5-FU concentration 24 h after treatment, and the cell cycle recovered by 72 h. G_2/M phase cells disappeared in the highest dose 24 h after treatment.

WTK-1 cells also underwent no cell cycle changes 0.5 h after treatment. Cells were arrested in S phase 24 h after treatment but recovered by 48 h in all concentration

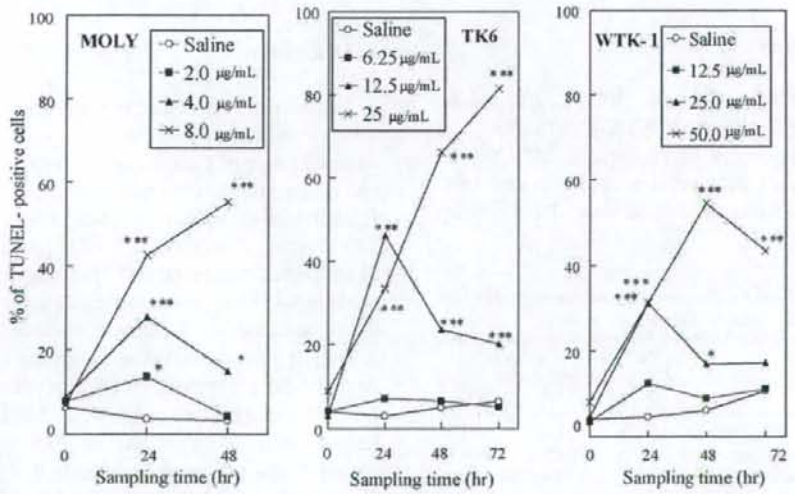

Fig. 3. The percentage of TUNEL-positive MOLY, TK6, and WTK-1 cells after 5-FU treatment. For analysis of apoptosis, 10,000 cells were analyzed at 0.5, 24, and 48 h (all cell lines) or at 72 h (TK6 and WTK-1 cells) after 5-FU treatment. 5-FU increased the ratio of apoptotic cells 24 h after treatment in all cell lines. The ratio of apoptotic cells was the highest in TK6 cells. The levels of apoptosis in MOLY and WTK-1 cells were similar at the same level of cytotoxicity. *$P < 0.05$; ***$P < 0.001$ (relative to saline control); χ^2-test.

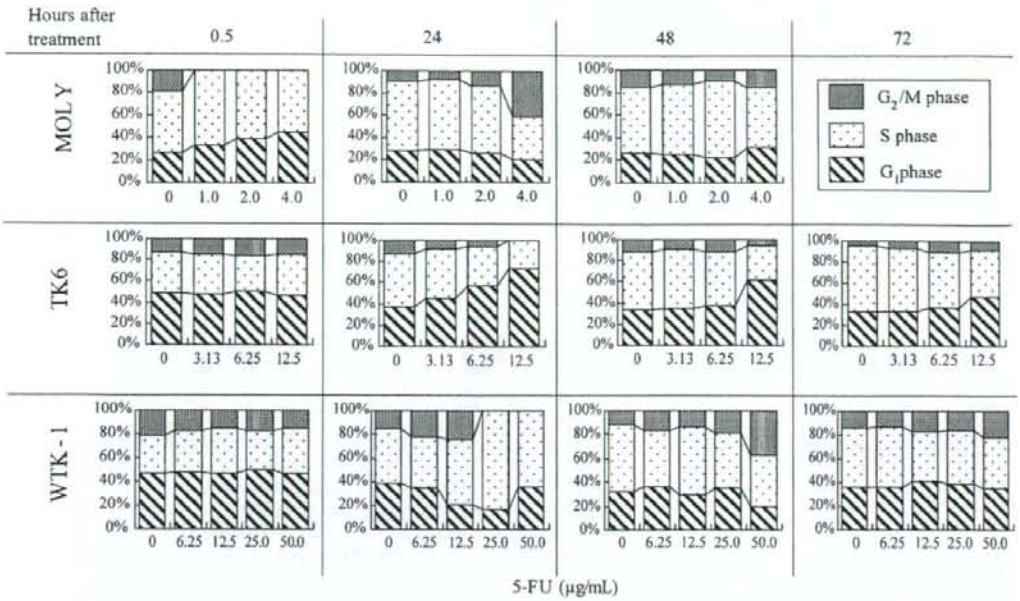

Fig. 4. Time course of cell cycle distribution in MOLY, TK6, and WTK-1 cells following 5-FU treatment. For analysis of cell cycle distribution, 10,000 cells were analyzed at 0.5, 24, and 48 h (all cell lines) or at 72 h (TK6 and WTK-1 cells) after 5-FU treatment. In MOLY cells, no G_2/M phase cells were evident 0.5 h after treatment. At 24 h, the cell cycle delayed at 4.0 μg/mL 5-FU. The cell cycle returned to normal 48 h after treatment. In TK6 and WTK-1 cells, there was no cell cycle change 0.5 h after treatment. G_2/M phase cells disappeared in the highest dose (TK6 cells) and in the higher 2 doses (WTK-1 cells) 24 h after treatment. In TK6 cells, G1 phase cells accumulated in direct proportion to 5-FU concentration 24 h after treatment, and the cell cycle recovered by 72 h. In WTK-1 cells, cells were arrested in S phase 24 h after treatment but recovered by 48 h in all concentration groups.

groups. G_2/M phase cells disappeared in the higher two doses 24 h after treatment.

3.6. Enzyme activity and TS content

Table 1 shows TS content and DPD, OPRT, and TP activity in MOLY, TK6, and WTK-1 cells. The cell lines did not differ much in TS content. MOLY cells showed the highest OPRT activity and the lowest DPD and TP activity. TK6 cells showed lower DPD activity than WTK-1 cells, but the TS content was higher in TK6 cells than in WTK-1 cells.

Table 1
The content and activities of 5-FU related metabolism and catabolism enzymes in MOLY, TK6, and WTK-1 cells

Enzyme (units)	Cell lines		
	MOLY	TK6	WTK-1
TS contents (pmol/min/mg protein)	0.726	1.284	0.573
OPRT activity (pmol/min/mg)	171.8	21.5	39.6
DPD activity (pmol/min/mg)	0.28	10.11	34.97
TP activity (pmol/min/mg)	ND	20.58	16.08

Abbr.: TS, thymidylate synthetase; OPRT, orotate phosphoribosyl transferase; DPD, dihydrouracil dehydrogenase; TP, thymidine phosphorylase; ND, not detected.

4. Discussion

In general, X-rays and mutagens induce more cytotoxicity and/or a higher frequency of apoptosis in cells with wild type p53 and a higher frequency of mutations and chromosomal aberrations in cells with mutant p53 [3]. Because the mutated p53 site in both MOLY (codon 170 of exon 5) and WTK-1 cells (codon 237 of exon 7) are part of the central DNA binding domain [13], and because 5-FU was positive in the *Tk* assay in MOLY cells [5,30], we expected 5-FU to be positive in WTK-1 cells as well. It was not, however, indicating that 5-FU mutagenicity did not depend on p53 function. Furthermore, 5-FU was much more cytotoxic in MOLY cells, which have a mutant p53 gene, than in TK6 cells, which have the wild type p53 gene, indicating that 5-FU cytotoxicity also did not depend on p53 function. At the same level of cytotoxicity, the level of apoptosis was similar in MOLY and WTK-1 cells, suggesting that the induction of apoptosis cells was not directly related to 5-FU muta-

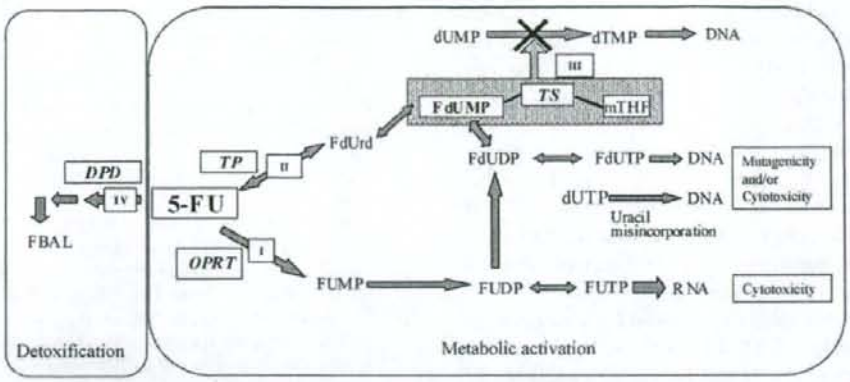

Fig. 5. The intracellular metabolism and catabolism of 5-FU. (I) 5-FU is metabolized by OPRT to FUMP. (II) 5-FU is metabolized to FdUrd by TP, but TP can convert FdUrd to 5-FU. (III) When 5-FU is converted to the active form of 5-FU, FdUMP, it forms complex with TS and 5,10-methylene tetrahydrofolate, and competitively inhibits DNA synthesis. (IV) 5-FU is detoxicated by DPD to FBAL. Thymidine depletion is a trigger for DSBs. DSBs are caused by the misincorporation of dUTP and/or misrepair of the uracil-containing lesion. Furthermore, 5-FU induces DNA strand breaks by the direct incorporation of 5-fluoro-deoxyuridine triphosphate (FdUTP). 5-FU is also misincorporated to RNA, and show the cytotoxicity. Modified from IARC Monographs [26].

genicity in MOLY and WTK-1 cells. Cell cycle analysis showed no clear difference between MOLY cells and the human cell lines, suggesting that p53 gene status did not directly determine the sensitivity of the cells to 5-FU mutagenicity and cytotoxicity.

Fig. 5 shows the intracellular metabolism and detoxification of 5-FU. When 5-FU, a fluorinated pyrimidine base analogue, is converted to 5-fluoro-2-deoxyuridylate monophosphate (FdUMP), it complexes with TS and 5,10-methylene tetrahydrofolate (CH_2FH_4), and competitively inhibits DNA synthesis [23–26]. Thymidine depletion by TS inhibition induces a nucleotide pool imbalance and the misincorporation of dUTP and/or misrepair of the uracil-containing lesion, causing double strand DNA breaks (DSBs) [40–42]. Furthermore, the direct incorporation of 5-fluoro-deoxyuridine triphosphate (FdUTP) inhibits DNA synthesis [24]. Therefore, the intracellular metabolism of 5-FU to FdUMP or FdUTP leads to cytotoxic and mutagenic effects.

5-FU can be metabolized by OPRT to fluoro-uridylate monophosphate and detoxified by DPD to fluoro-β-alanine, or by TP to 5-fluoro-deoxyuridine (FdUrd) and, in turn, back to 5-FU (Fig. 5). In this study, DPD activity was lower and OPRT activity was higher in MOLY cells than in TK6 and WTK-1 cells, suggesting the efficient metabolism of 5-FU in MOLY cells, which could have led to the strong cytotoxic and mutagenic effects that we observed.

Only MOLY cells had no TP activity in this study. TP and OPRT are involved in the intracellular metabolism of 5-FU. Inhibition of OPRT decreases the incorporation of 5-FU into nucleotides and therefore its cytotoxicity, while TP has no effect [43]. Thus, the absence of TP activity in MOLY cells would have little effect on its sensitivity to 5-FU. TS content, on the other hand, was not associated with the cytotoxic and mutagenic effects of 5-FU.

From these findings, we conclude that the difference in DPD and OPRT activity in MOLY, TK6, and WTK-1 cells were responsible for differences in the cells' cytotoxic and mutagenic responses to 5-FU.

The relationship between sensitivity to 5-FU and the intracellular distribution of 5-FU metabolic enzymes indicate that differences in the way that human cells and animal cells metabolize a chemical can cause differences in mutagenicity test results. Thus, potentially mutagenic chemicals should be tested in human cells as well as animal cells.

Compared with WTK-1 cells, TK6 cells had much lower DPD activity, and 5-FU in TK6 cells was efficiently metabolized to FdUMP without being detoxified. But the TS content was higher in TK6 cells than in WTK-1 cells, so 5-FU metabolism would not be very different in TK6 and WTK-1 cells. In this study, however, 5-FU cytotoxicity was higher in TK6 cells than in WTK-1 cells, perhaps because 5-FU is more cytotoxic in p53 normal cells than in p53 mutant or null cells [15–17]. Our results suggest that if 5-FU metabolizing enzyme activity is equivalent, p53 status might influence 5-FU cytotoxicity. Indeed, p53 status might influence responses to cytotoxicity induced by 5-FU as it does to cytotoxicity induced by X-rays [3]. Furthermore 5-FU increased the

MN frequency in MOLY and WTK-1 cells but not TK6 cells, while the ratio of apoptosis cells induced by 5-FU was the highest in TK6 cells. In TK6 cells, the wild type p53 gene might suppress the induction of micronuclei by leading of DNA damaged cells to apoptosis. Thus, 5-FU mutagenicity might be influenced by also the p53 gene status. However, IC_{50} of RS0 was obviously lower and MF was obviously higher in MOLY cells than TK6 and WTK-1 cells. Therefore we suspected that the difference in 5-FU metabolism influenced to 5-FU cytotoxicity and mutagenicity more than the p53 gene status.

In summary, our study showing that MOLY cells were more sensitive than WTK-1 cells to 5-FU cytotoxicity and mutagenicity even though both have a mutated p53 gene suggested that those differences were attributable to differences in 5-FU metabolism rather than the p53 gene status.

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References

[1] T.J. Oberly, B.J. Bewsey, G.S. Probst, An evaluation of L5178YTK$^{+/-}$ mouse lymphoma forward mutation assay using 42 chemicals, Mutat. Res. 125 (1984) 291–306.

[2] P. Clay, M.F. Cross, Microwell mutation assays: evaluation of ethyl methanesulfonate, benzo[a]pyrene and benzidine using the tk locus in L5178Y mouse lymphoma cells, Mutagenesis 5 (Suppl.) (1990) 45–54.

[3] M. Honma, M. Hayashi, T. Sofuni, Cytotoxic and mutagenic responses to X-rays and chemical mutagens in normal and p53-mutated human lymphoblastoid cells, Mutat. Res. 374 (1997) 89–98.

[4] M. Honma, L.S. Zhang, M. Hayashi, K. Takeshita, Y. Nakagawa, N. Tanaka, T. Sofuni, Illegitimate recombination leading to allelic loss and unbalanced translocation in p53-mutated human lymphoblastoid cells, Mol. Cell. Biol. 17 (1997) 4774–4781.

[5] M. Honma, M. Hayashi, H. Shimada, N. Tanaka, S. Wakuri, T. Awogi, K.I. Yamamoto, N. Kodani, Y. Nishi, M. Nakadate, T. Sofuni, Evaluation of the mouse lymphoma tk assay (microwell method) as an alternative to the in vitro chromosomal aberration test, Mutagenesis 14 (1999) 5–22.

[6] D. Clive, J.F.S. Spector, Laboratory procedure assessing specific locus mutation at the TK locus in cultured L5178Y mouse lymphoma cells, Mutat. Res. 31 (1975) 17–29.

[7] P.M. Leong-Morgenthaler, C.O.H. Velt, E. Jaccaud, R.J. Turesky, Mutagenicity of 2-amino-3-methylimidazo[4,5-f]quinoline in human lymphoblastoid cells, Carcinogenesis 19 (1998) 1749–1754.

[8] Y.Y. Chuang, Q. Chen, J.P. Brown, J.M. Sedivy, H.L. Liber, Radiation-induced mutations at the autosomal thymidine kinase locus are not elevated in p53-null cells, Cancer Res. 59 (1999) 3073–3076.

[9] R.F. Branda, J.P. O'Neill, E.M. Brooks, L.M. Trombley, J.A. Nicklas, The effect of folate deficiency on the cytotoxic and mutagenic responses to ethyl methanesulfonate in human lymphoblastoid cell lines that differ in p53 status, Mutat. Res. 473 (2001) 51–71.

[10] L. Bassi, M. Carloni, E. Fonti, N.P. de la Pena, R. Meschini, F. Palitti, Pifithrin-alpha, an inhibitor of p53, enhances the genetic instability induced by etoposide (VP16) in human lymphoblastoid cells treated in vitro, Mutat. Res. 499 (2002) 163–176.

[11] S.A. Amundson, F. Xia, K. Wolfson, H.L. Liber, Different cytotoxic and mutagenic responses induced by X-rays in two human lymphoblastoid cell lines derived from a single donor, Mutat. Res. 286 (1993) 233–241.

[12] F. Xia, X. Wang, Y.H. Wang, N.M. Tsang, D.W. Yandell, K.T. Kelsey, H.L. Liber, Altered p53 status correlates with differences in sensitivity to radiation-induced mutation and apoptosis in two closely related human lymphoblast lines, Cancer Res. 55 (1995) 12–15.

[13] S.M. Morris, A role for p53 in the frequency and mechanism of mutation, Mutat. Res. 511 (2002) 45–62.

[14] J.L. Schwartz, R. Jordan, B.A. Sedita, M.J. Swenningson, J.P. Banath, P.L. Olive, Different sensitivity to cell killing and chromosome mutation induction by gamma rays in two human lymphoblastoid cell lines derived from a single donor: possible role of apoptosis, Mutagenesis 10 (1995) 227–233.

[15] D. Arango, G.A. Corner, S. Wadler, P.J. Catalano, L.H. Augenlicht, c-myc/p53 interaction determines sensitivity of human colon carcinoma cells to 5-fluorouracil in vitro and in vivo, Cancer Res. 61 (2001) 4910–4915.

[16] J.L. Grem, K.D. Danenberg, K. Behan, A. Parr, L. Young, P.V. Danenberg, D. Nguyen, J. Drake, A. Monks, C.J. Allegra, Thymidine kinase, thymidylate synthase, and dihydropyrimidine dehydrogenase profiles of cell lines of the National Cancer Institute's Anticancer Drug Screen, Clin. Cancer Res. 7 (2001) 999–1009.

[17] D.B. Longley, J. Boyer, W.L. Allen, T. Latif, P.R. Ferguson, P.J. Maxwell, U. McDermott, M. Lynch, D.P. Harkin, P.G. Johnston, The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates, Cancer Res. 62 (2002) 2644–2649.

[18] S.K. Greenwood, M.J. Armstrong, R.B. Hill, C.I. Bradt, T.E. Johnson, C.A. Hilliard, S.M. Galloway, Fewer chromosome aberrations and earlier apoptosis induced by DNA synthesis inhibitors, a topoisomerase II inhibitor or alkylating agents in human cells with normal compared with mutant p53, Mutat. Res. 401 (1998) 39–53.

[19] T. Allio, E.M. Donner, R.J. Preston, A comparison of the roles of p53 mutation and AraC inhibition in the enhancement of bleomycin-induced chromatid aberrations in mouse and human cells, Mutat. Res. 447 (2000) 227–237.

[20] T. Allio, R.J. Preston, Increased sensitivity to chromatid aberration induction by bleomycin and neocarzinostatin results from alterations in a DNA damage response pathway, Mutat. Res. 453 (2000) 5–15.

[21] L.S. Zhang, M. Honma, M. Hayashi, T. Suzuki, A. Matsuoka, T. Sofuni, A comparative study of TK6 human lymphoblastoid and L5178Y mouse lymphoma cell lines in the in vitro micronucleus test, Mutat. Res. 347 (1995) 105–115.

[22] R.D. Storer, A.R. Kraynak, T.W. McKelvey, M.C. Elia, T.L. Goodrow, J.G. DeLuca, The mouse lymphoma L5178Y $Tk^{+/-}$ cell line is heterozygous for a codon 170 mutation in the p53 tumor suppressor gene, Mutat. Res. 373 (1997) 157–165.

[23] A.A. Piper, R.M. Fox, Biochemical basis for the differential sensitivity of human T- and B-lymphocyte lines to 5-fluorouracil, Cancer Res. 42 (1982) 3753–3760.

[24] R.B. Diasio, B.E. Harris, Clinical pharmacology of 5-fluorouracil, Clin. Pharmacokinet. 16 (1989) 215–237.

[25] D.M. Thomas, J.R. Zalcberg, 5-fluorouracil: a pharmacological paradigm in the use of cytotoxics, Clin. Exp. Pharmacol. Physiol. 25 (1998) 887–895.

[26] 5-fluorouracil, IARC Monogr. Eval. Carcinogen. Risk Chem. Hum. 26 (1981) 217–235.

[27] L. Griffiths, I.J. Stratford, Platelet-derived endothelial cell growth factor thymidine phosphorylase in tumor growth and response to therapy, Br. J. Cancer 76 (1997) 689–693.

[28] N. Yajima, K. Kondo, K. Morita, Reverse mutation tests in *Salmonella typhimurium* and chromosomal aberration tests in mammalian cells in culture on fluorinated pyrimidine derivatives, Mutat. Res. 88 (1981) 241–254.

[29] A. Ohuchida, A. Furukawa, J. Yoshida, M. Watanabe, F. Aruga, Y. Miwa, K. Shinkawa, N. Kinae, Micronucleus assays on 5-fluorouracil and 6-mercaptopurine with mouse peripheral blood reticulocytes, Mutat. Res. 278 (1992) 139–143.

[30] T. Sofuni, M. Honma, M. Hayashi, H. Shimada, N. Tanaka, S. Wakuri, T. Awogi, K.I. Yamamoto, Y. Nishi, M. Nakadate, Detection of in vitro clastogens and spindle poisons by the mouse lymphoma assay using the microwell method: interim report of an international collaborative study, Mutagenesis 11 (1996) 349–355.

[31] 5-fluorouracil, IARC Monogr. Eval. Carcinogen. Risk Chem. Hum. (Suppl. 6) (1987) 316–318.

[32] T. Matsushima, M. Hayashi, A. Matsuoka, M. Ishidate, K.F. Miura, H. Shimizu, Y. Suzuki, K. Morimoto, H. Ogura, K. Mure, K. Koshi, T. Sofuni, Validation study of the in vitro micronucleus test in a Chinese hamster lung cell line (CHL/IU), Mutagenesis 14 (1999) 569–580.

[33] Y. Gavrieli, Y. Sherman, S.A. Ben-Sasson, Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation, J. Cell Biol. 119 (1992) 493–501.

[34] M. Carbonari, M. Cibati, M. Fiorilli, Measurement of apoptotic cells in peripheral blood, Cytometry 22 (1995) 161–167.

[35] C.P. Spears, A.H. Shahinian, R.G. Moran, C. Heidelberger, T.H. Corbett, In vivo kinetics of thymidylate synthetase inhibition of 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas, Cancer Res. 42 (1982) 450–456.

[36] T. Takechi, J. Uchida, F.A.M. Fukushima, Enhancing 5-fluorouracil cytotoxicity by inhibiting dihydropyrimidine dehydrogenase activity with uracil in human tumor cells, Int. J. Oncol. 11 (1997) 1041–1044.

[37] F.N. Naguib, M.H. el Kouni, S. Cha, Enzymes of uracil catabolism in normal and neoplastic human tissues, Cancer Res. 45 (1985) 5405–5412.

[38] G.J. Peters, E. Laurensse, A. Leyva, J. Lankelma, H.M. Pinedo, Sensitivity of human, murine, and rat cells to 5-fluorouracil and 5′-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes, Cancer Res. 46 (1986) 20–28.

[39] Y. Maehara, H. Nakamura, Y. Nakane, K. Kawai, M. Okamoto, S. Nagayama, T. Shirasaka, S. Fujii, Activities of various enzymes of pyrimidine nucleotide and DNA syntheses in normal and neoplastic human tissues, Gann 73 (1982) 289–298.

[40] H.A. Ingraham, B.Y. Tseng, M. Goulian, Nucleotide levels and incorporation of 5-fluorouracil and uracil into DNA of cells treated with 5-fluorodeoxyuridine, Mol. Pharmacol. 21 (1982) 211–216.

[41] N.J. Curtin, A.L. Harris, G.W. Aherne, Mechanism of cell death following thymidylate synthase inhibition: 2′-deoxyuridine-5′-triphosphate accumulation, DNA damage, and growth inhibition following exposure to CB3717 and dipyridamole, Cancer Res. 51 (1991) 2346–2352.

[42] R.D. Ladner, The role of dUTPase and uracil-DNA repair in cancer chemotherapy, Curr. Protein Pept. Sci. 2 (2001) 361–370.

[43] M. Fukushima, Y. Murakami, N. Suzuki, K. Aiba, The analysis of the innate pathways of 5-fluorouracil phosphorylation in human gastrointestinal cancer cell lines in vitro and in vivo, Oncol. Rep. 4 (1997) 1189–1194.