

Table 5. Continued.

Probe Set ID	Gene Symbol	Gene ID	Fold change		Gene Title
			6W	12W	
1370583_s_at	Abcb1 /// Abcb1a	AY082609	3.6	4.1	ATP-binding cassette, sub-family B (MDR/TAP), member 1 /// ATP-binding cassette, sub-family B (MDR/TAP), member 1A
1368147_at	Dusp1	BE110108		4.1	dual specificity phosphatase 1
1388721_at	Hspb8	BG380282		4.1	heat shock 22kDa protein 8
1368213_at	Por	AI407454		4.1	P450 (cytochrome) oxidoreductase
1372523_at	Gclc	AA892770	2.2	4.0	glutamate-cysteine ligase, catalytic subunit
1386995_at	Btg2	BI288701		4.0	B-cell translocation gene 2, anti-proliferative
1386922_at	Ca2	AI408948	4.5	4.0	carbonic anhydrase 2
1387283_at	Mx2	NM_134350	2.0	3.9	myxovirus (influenza virus) resistance 2
1372389_at	Ier2	BF420059		3.6	immediate early response 2
1371237_a_at	Mt1a	AF411318	4.5	3.6	metallothionein 1a
1372261_at	---	AI409067	2.7	3.5	Transcribed locus
1373473_a_at	Nap111	BM386384	2.1	3.5	nucleosome assembly protein 1-like 1
1367733_at	Ca2	NM_019291	3.2	3.5	carbonic anhydrase 2
1373810_at	Pla2g12a_predicted	BF284175	3.6	3.5	phospholipase A2, group X1IA (predicted)
1377092_at	---	BF389682		3.4	Transcribed locus
1388622_at	Nol5a	AW535890		3.4	nucleolar protein 5A
1389573_at	Chac1_predicted	AI170665		3.4	ChaC, cation transport regulator-like 1 (E. coli) (predicted)
1375895_at	---	BI275908		3.3	CDNA clone IMAGE:7302535, with apparent retained intron
1368332_at	Gbp2	NM_133624		3.3	guanylate nucleotide binding protein 2
1369061_at	Gsr	NM_053906	2.2	3.3	glutathione reductase

Fold changes in 6-week-old rats are also shown for values higher than 2.

which are Cyp3A inducers, increased APAP hepatotoxicity (Jaw and Jeffery, 1993; Madhu *et al.*, 1993; Jue *et al.*, 2002; Guo *et al.*, 2004). Therefore, it would be reasonable to assume that the difference in basal expression levels of Cyp3A13 between young and adult rats accounts for the difference in susceptibility to APAP toxicity. Cyp2E1 is another enzyme responsible for biotransformation, i.e., inhibition of Cyp2E1 decreased the production of a reactive metabolite in the liver microsome from humans (Hazai *et al.*, 2002), although this enzyme did not show an age-difference. Based on the present results, participation of CYP3A is considered to be larger than that of CYP2E in the rat.

GSTYc2 was one isoform of glutathione *S*-transferase, and was known to play an important role in aflatoxin detoxification (Buetler and Eaton, 1992; Hayes *et al.*, 1992 and 1994). Although the role of GSTYc2 in APAP detoxification has not been investigated, it could have some role as GST conjugation, a key detoxification pathway of the reactive intermediate

of APAP.

When the total of the GST and SH-contents in the liver were measured, they were found to be higher in adult than in young under the control condition, while their reduction by APAP-treatment was larger in adult than in young. This could be explained by our assumption from gene expression analysis that the production of active metabolite is higher and the rate of the supply of the detoxification enzyme is lower in adult rats.

The age-related difference in the number of mobilized genes at 24 hr after APAP-treatment was considered to be correlated with the phenotype. Namely, most of these changes in the expression reflected the results of pathological changes. When the precise analysis of the genes showing age-related difference was made in terms of time- and dose-related responses, another view emerged. Among the genes with age-related difference at 24 hr after APAP-treatment, there were not only genes with differing levels of expression but also differences in the response time.

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Table 6. List of genes that showed expression of less than 0.3-fold of control by APAP at 24 hr in 12-week-old rats.

Probe Set ID	Gene Symbol	Gene ID	Fold change		Gene Title
			6W	12W	
1371412_a_at	Nrep	BE107450	0.25	0.06	Neuronal regeneration related protein
1386977_at	Ca3	NM_019292	0.20	0.08	carbonic anhydrase 3
1374251_at	---	AA893192		0.11	Transcribed locus
1367896_at	Ca3	AB030829		0.13	carbonic anhydrase 3
1369864_a_at	Sds	NM_053962	0.47	0.17	serine dehydratase
1387665_at	Bhmt	U96133	0.40	0.18	betaine-homocysteine methyltransferase
1374244_at	LOC501038	AI411141	0.35	0.18	Ab2-060
1376976_at	Sectm1	AI009823		0.18	secreted and transmembrane 1
1373686_at	Serpina6_mapped	AA893495	0.42	0.19	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6 (mapped)
1389681_at	Pvr12	BI296388	0.24	0.21	Poliovirus receptor-related 2 (herpesvirus entry mediator B)
1368171_at	Lox	NM_017061	0.33	0.21	lysyl oxidase
1374531_at	---	AA926305		0.22	Transcribed locus
1368826_at	Comt	NM_012531		0.22	catechol-O-methyltransferase
1393221_at	RGD1564865_predicted	AA866264		0.23	similar to 20-alpha-hydroxysteroid dehydrogenase (predicted)
1368458_at	Cyp7a1	NM_012942	0.45	0.23	cytochrome P450, family 7, subfamily a, polypeptide 1
1368172_a_at	Lox	BI304009		0.25	lysyl oxidase
1373778_at	---	BE349670	0.32	0.26	Transcribed locus
1368270_at	Apobec1	NM_012907		0.26	apolipoprotein B editing complex 1
1373814_at	RGD1310066	BI291270		0.26	similar to mKIAA1002 protein
1398759_at	Tgfb1i4	NM_013043	0.34	0.27	transforming growth factor beta 1 induced transcript 4
1388583_at	Cxcl12	BF283398	0.42	0.27	chemokine (C-X-C motif) ligand 12
1368778_at	Slc6a6	NM_017206	0.34	0.27	solute carrier family 6 (neurotransmitter transporter, taurine), member 6
1368543_at	Nox4	NM_053524		0.27	NADPH oxidase 4
1375560_at	RGD1310475_predicted	AA945579		0.28	similar to RIKEN cDNA 0610010D20 (predicted)
1388426_at	Srebf1	BF398848	0.50	0.28	sterol regulatory element binding factor 1
1387053_at	Fmol	NM_012792		0.28	flavin containing monooxygenase 1
1375216_at	Pvr12	AA850909	0.31	0.28	poliovirus receptor-related 2 (herpesvirus entry mediator B)
1387263_at	Pklr	NM_012624		0.28	pyruvate kinase, liver and red blood cell
1374493_at	---	H35017		0.29	Transcribed locus
1369664_at	Avpr1a	NM_053019	0.36	0.30	arginine vasopressin receptor 1A
1377015_at	Me3_predicted	BF395080	0.43	0.30	Malic enzyme 3, NADP(+)-dependent, mitochondrial (predicted)
1371137_at	Acox2	X95189		0.30	acyl-Coenzyme A oxidase 2, branched chain
1372684_at	Smtn	AW435036		0.30	smoothelin
1387123_at	Cyp17a1	NM_012753	0.30	0.30	cytochrome P450, family 17, subfamily a, polypeptide 1
1367729_at	Oat	NM_022521	0.30	0.30	ornithine aminotransferase
1374625_at	Hes6	AI176616	0.47	0.30	hairy and enhancer of split 6 (Drosophila)
1373975_at	LOC368066	AI232716		0.30	similar to thioether S-methyltransferase

Fold changes in 6-week-old rats are also shown for values lower than 0.5.

Especially, genes related to defensive responses to stress, e.g., heat shock protein, Hmox-1, Gadd45, and thioredoxin reductase were markedly induced by APAP in young rats at 6 or 9 hr after treatment, whereas adult rats did not respond as yet. This is considered to be due to the age-related difference in the toxicokinetics, i.e., young rats responded to APAP in the early stage of exposure because of the low SH level but recovered at 24 hr because of the high detoxification capacity.

In the case of CCl₄, its active metabolite is reported to be produced by CYP2E1 (Parkinson, 2001) and this fits the present observation that neither the

expression of CYP2E1 nor hepatotoxicity of CCl₄ showed a difference between the ages. Although GSH conjugation should play an important role in their detoxification, the participation of GSTYc2 is considered to be less important for CCl₄ in rats. It has been reported that both *N*-acetylation and CYP2E1 are involved in hepatotoxicity of INH (Parkinson, 2001). In the present study, no age-related difference was found in the expression of *N*-acetylating enzymes and CYP2E1, in accordance with the same extent of hepatotoxicity of INH for both ages. However, one should be careful in these conclusions, since phenotypical changes by INH and CCl₄ were minimal in the present

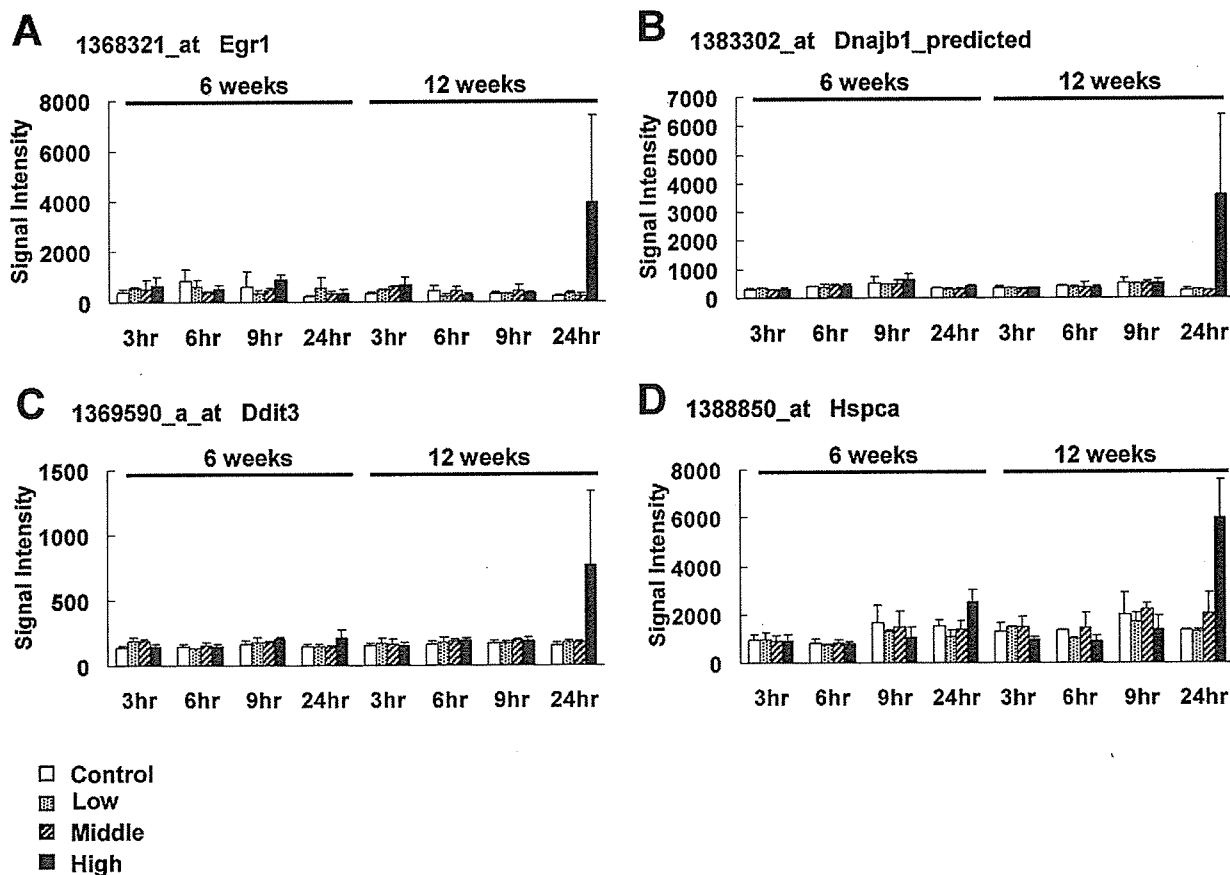


Fig. 3. Time- and dose-dependent effects of APAP on expression of Egr1 (A), Dnajb1 (predicted) (B), Ddit3 (C), and Hspca (D). Rats 6- or 12-week-old were treated with 50 (low), 300 (middle) and 1000 (high) mg/kg of APAP and sacrificed at 3, 6, 9, and 24 hr after treatment. Gene expression in liver was analyzed by using GeneChip[®] RAE230A probe arrays and the signals obtained by Affymetrix Microarray Suite version 5.0 were normalized by using the mean value of each chip adjusted to an arbitrary value of 500 and then expressed as mean \pm SD (N=3). Note that the expression of these genes was markedly increased by APAP-treatment at 24 hr in adult (12-week-old) rats.

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condition, which was optimized for their repeated administration for up to 28 days in our standard protocol.

The TGP database has huge gene expression data as well as the traditional toxicity study parameters, currently for over 100 chemicals. Although the main purpose of the project is to predict toxicity in the early stage of drug development, it is also useful for drawing some mechanistic insight in observed toxicity. In the present study, it would have been impossible to pick up such genes responding in a different time course with age, unless a multi-time, multi-dose protocol was employed. In general, dose or time points tend to be limited in the collection of toxicological data by

microarray due to the cost. The present study has clearly showed the advantage of our protocol with enough numbers of data points. This promises a good use for our database now being created in the project.

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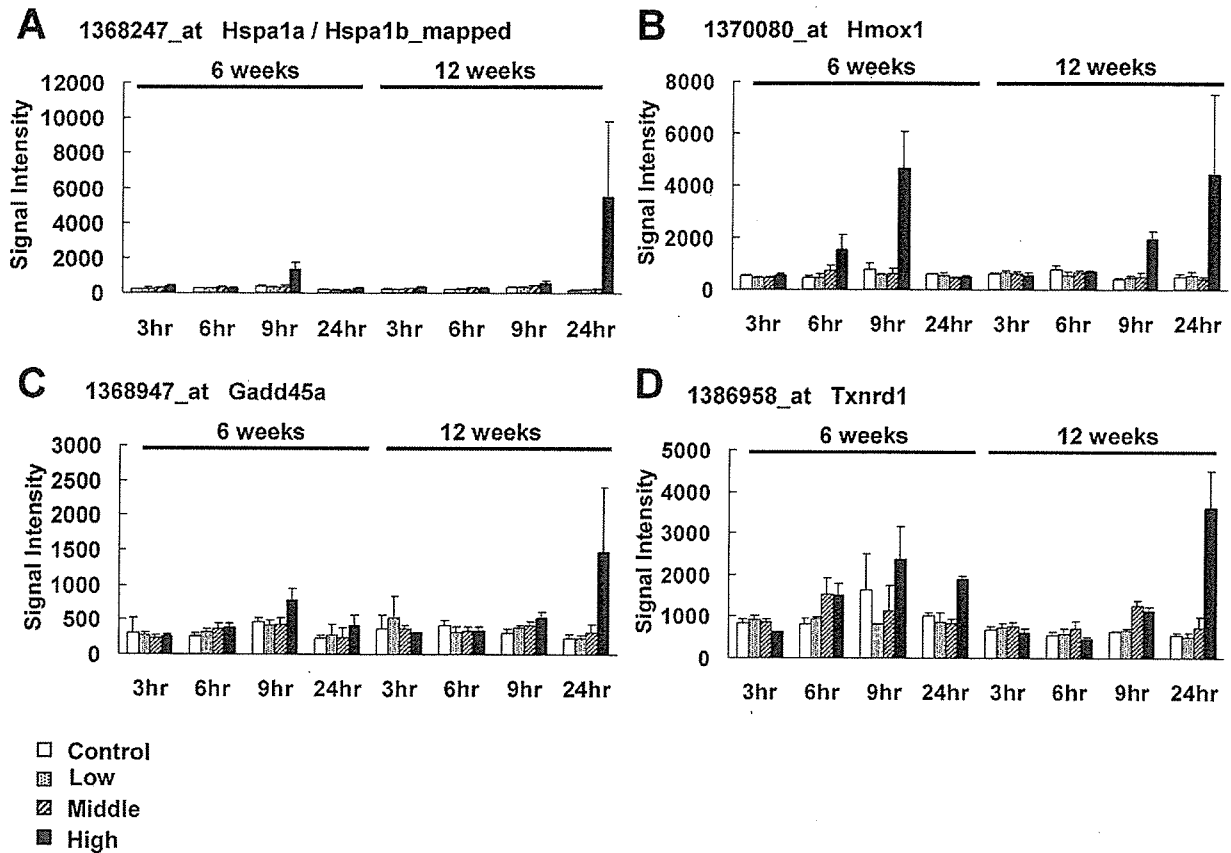


Fig. 4. Time- and dose-dependent effects of APAP on expression of Hsp a1a/1b (A), Hmox-1 (B), Gadd45 (C), and Txnrd (D). Rats 6- or 12-week-old were treated with 50 (low), 300 (middle) and 1000 (high) mg/kg of APAP and sacrificed at 3, 6, 9, and 24 hr after treatment. Gene expression in liver was analyzed by using GeneChip[®] RAE230A probe arrays and the signals obtained by Affymetrix Microarray Suite version 5.0 were normalized by using the mean value of each chip adjusted to an arbitrary value of 500 and then expressed as mean \pm SD (N=3). Note that the induction of these genes by APAP at 24 hr was larger in adult (12-week-old) than young (6-week-old) rats, whereas the young rat responded to APAP earlier.

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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.

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Keywords: Acrylamide; Glycidamide; 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 lymphoblastoid 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 μ g/ml sodium pyruvate, 100 U/ml penicillin and 100 μ g/ml streptomycin and maintained at 10^5 to 10^6 cells/ml at 37 °C in a 5% CO₂ 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 μ g/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 $\mu\text{g/ml}$ 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 re-fed 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-

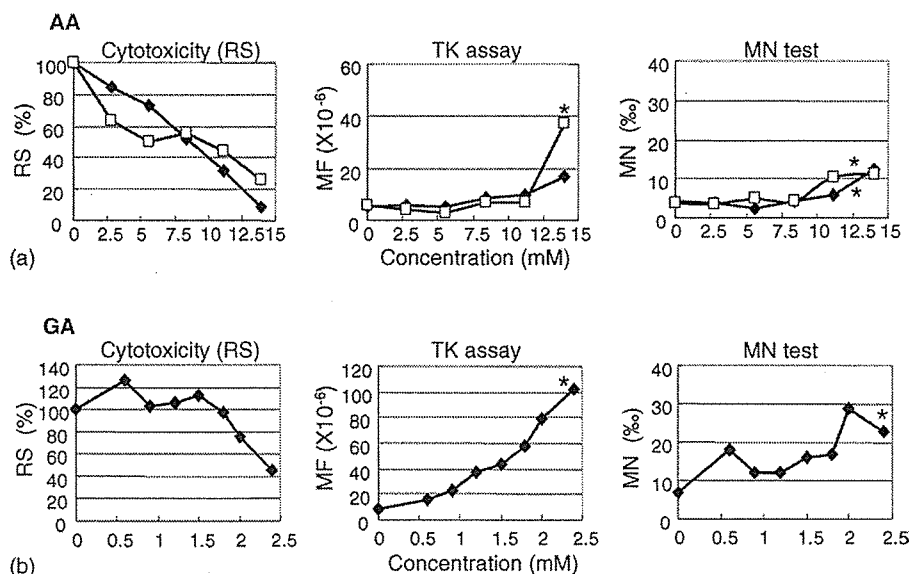


Fig. 1. Cytotoxic (relative survival, RS), genotoxic responses (TK assay and MN test) of TK6 cells treated with AA (a) or GA (b) for 4 h without metabolic activation. The AA experiment was repeated to confirm the result because of the weak genotoxicity. Closed and open symbols are first and second experiment, 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

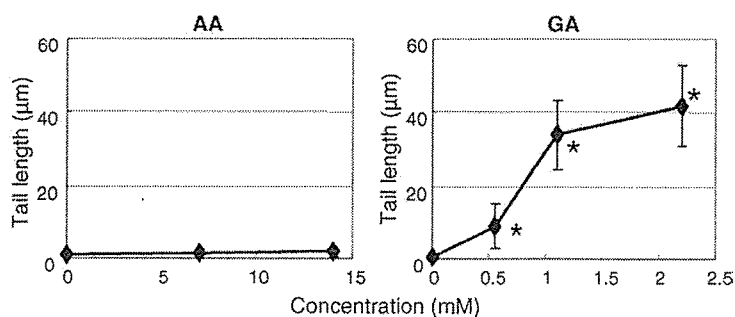


Fig. 2. COM assay results in TK6 cells treated with AA or GA for 4 h without metabolic activation. Asterisk (*) statistically significant in the Dunnett's tests ($P < 0.05$).

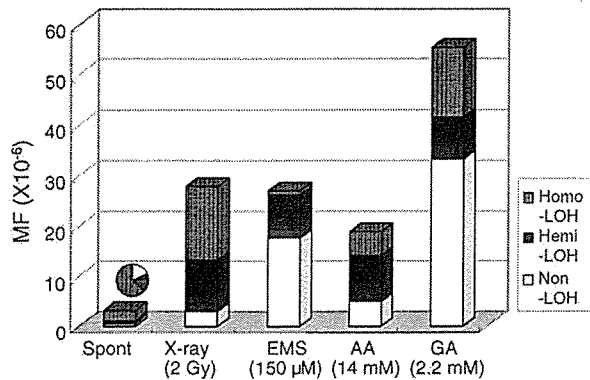


Fig. 3. Frequency and spectra of TK mutations in spontaneous and X-ray-induced (2 Gy), EMS-induced (150 μM, 4 h), AA-induced (14 mM, 4 h) and GA-induced (2.2 mM, 4 h) TK mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of normally growing (NG) and slowly 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

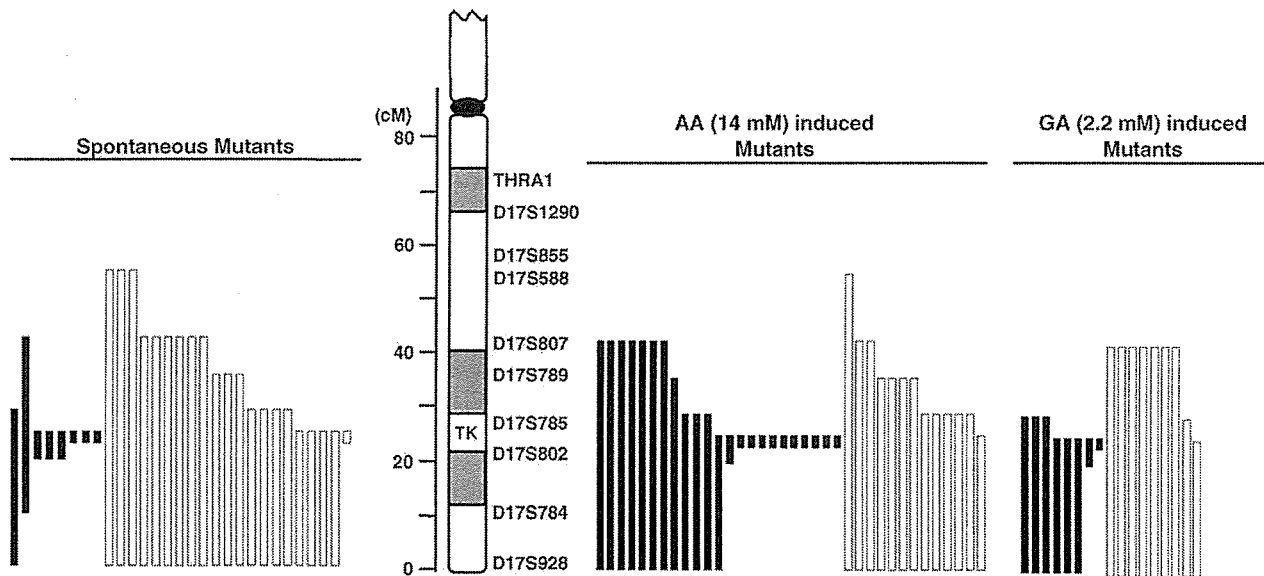


Fig. 4. The extent of LOH in spontaneous, AA-induced and GA-induced LOH mutants from TK6 cells. We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human *TK* locus maps to 17q23.2. Open and closed bars represent homo-LOH and hemi-LOH, respectively. The length 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].

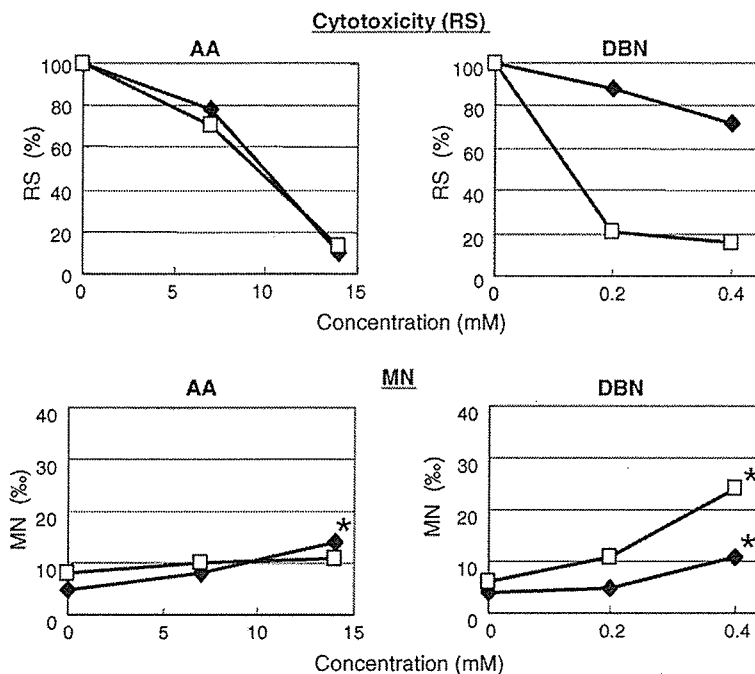


Fig. 5. Cytotoxicity (RS) and MN induction in TK6 cells treated with AA or DBN for 4 h in the presence (open symbol) or absence (closed symbol) of rat liver S9. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

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-homologous end-joining [13]. Radiation-induced double strand breaks are repaired by non-homologous 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 *N7*-(2-carbamoyl-2-hydroxyethyl) guanine and *N3*-(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 dihydroxy 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 BlueTM 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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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 IC₅₀ 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.

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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 heterozygosity

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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 G₁ 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 × 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 methane-sulfonate (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. [6-¹⁴C]-5-FU (1.85 GBq/mmol) and [6-³H]-FdUMP (625 GBq/mmol) were purchased from Moravak 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% CO₂ 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 [6-³H]-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 [6-³H]-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 MgCl₂, 0.02 mM [6-¹⁴C]-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 ([6-¹⁴C]-5-FU plus the catabolism fraction).

OPRT activity was determined according to the modified method of Peters et al. [38] using [6-¹⁴C]-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 MgCl₂, 4 mM PRPP, and 0.02 mM [6-¹⁴C]-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-deoxyuridylylate monophosphate) to the total radioactivity ([6-¹⁴C]-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 MgCl₂, 4 mM deoxyribose-1-phosphate, 10 mM ATP, and 0.02 mM [6-¹⁴C]-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-fluoro-deoxyuridine) to total radioactivity ([6-¹⁴C]-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 RSO was 2.4 $\mu\text{g}/\text{mL}$ in MOLY cells, 15.4 $\mu\text{g}/\text{mL}$ in TK6 cells, and 30.6 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ and quadrupling it at 4.0 $\mu\text{g}/\text{mL}$ (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 $\mu\text{g}/\text{mL}$ ($P=0.0003$) and 14.5 times the control value at 4.0 $\mu\text{g}/\text{mL}$ ($P<0.0001$) 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 $\mu\text{g}/\text{mL}$ ($P=0.0001$). 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 $\mu\text{g}/\text{mL}$.

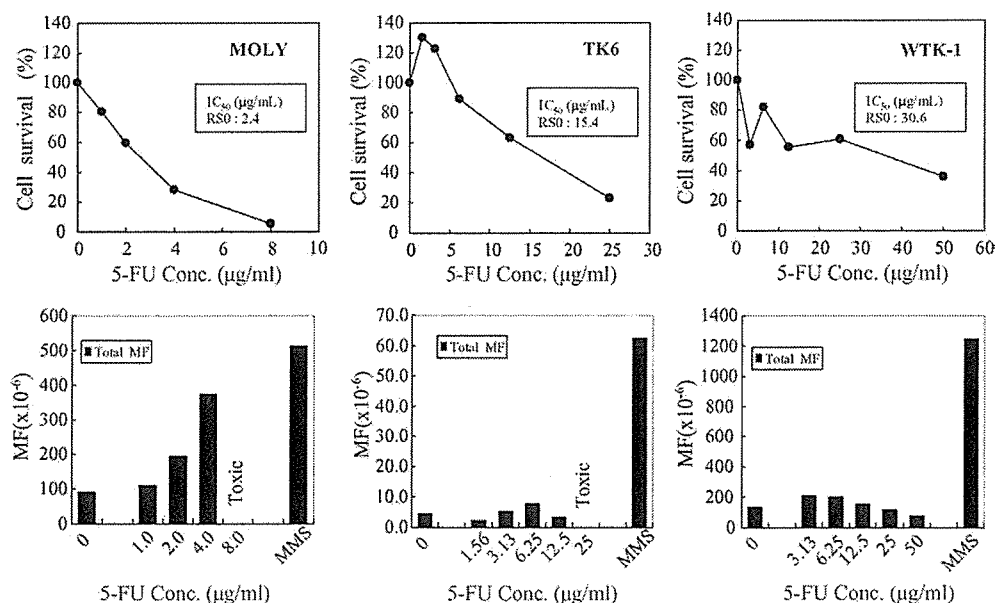


Fig. 1. The mutation frequency and cytotoxicity in tk assay after 5-FU treatment in MOLY, TK6 and WTK-1 cells. The percentage of cell survival (RSO, line graphs) and total MF (bar graphs) after 5-FU treatment (MOLY cells for 3 h and the human cell lines for 4 h) were shown. 5-FU was cytotoxic in all 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.

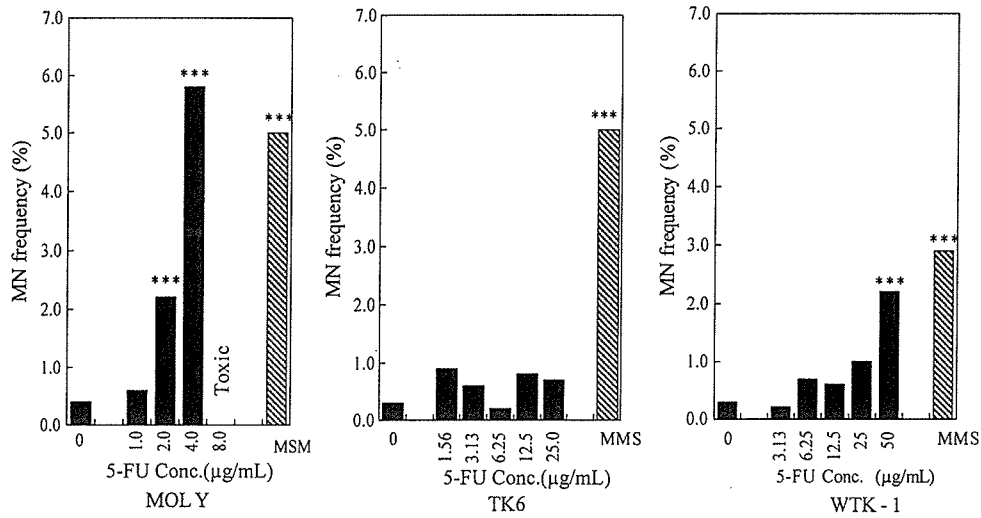


Fig. 2. The frequency of cells with micronuclei in MOLY, TK6 and WTK-1 cells after 5-FU treatment. The frequency of cells with MN (MN frequency) 48 h after 5-FU treatment was shown. One thousand cells from each treatment group were observed and the number with MN was counted. 5-FU significantly increased MN frequency in comparison with the control value at 2.0 ($P = 0.0003$) and 4.0 $\mu\text{g/mL}$ ($P < 0.0001$) in MOLY cells. 5-FU increased MN frequency only at 50 $\mu\text{g/mL}$ ($P = 0.0001$) 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

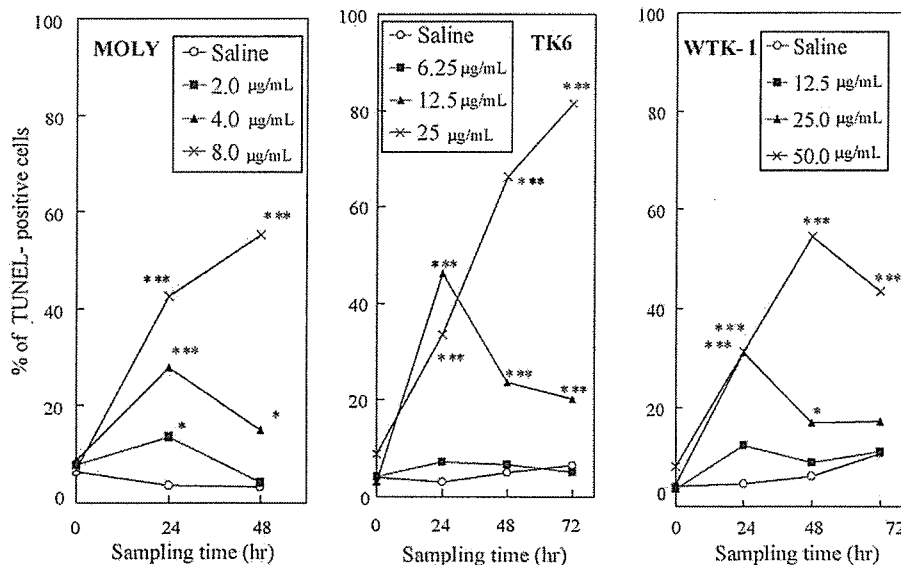


Fig. 3. The percentage of TUNEL-positive MOLY, TK6, and WTK-1 cells after 5-FU treatment. For analysis of apoptosis, 10,000 cells were analyzed at 0.5, 24, and 48 h (all cell lines) or at 72 h (TK6 and WTK-1 cells) after 5-FU treatment. 5-FU increased the ratio of apoptotic cells 24 h after treatment 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.

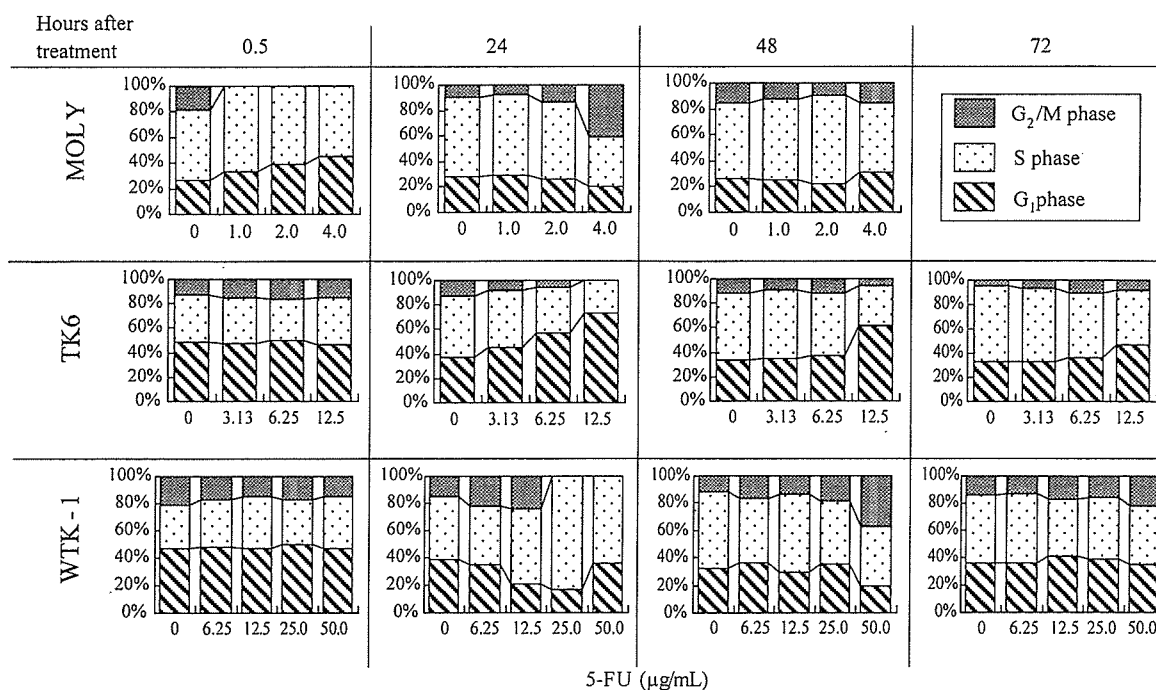


Fig. 4. Time course of cell cycle distribution in MOLY, TK6, and WTK-1 cells following 5-FU treatment. For analysis of cell cycle distribution, 10,000 cells were analyzed at 0.5, 24, and 48 h (all cell lines) or at 72 h (TK6 and WTK-1 cells) after 5-FU treatment. In MOLY cells, no G₂/M phase cells were 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. G₂/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. G₂/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

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.

than WTK-1 cells, but the TS content was higher in TK6 cells than in WTK-1 cells.

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-