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Use of Metabolically Competent Human Hepatoma Cells for the Detection of Mutagens and Antimutagens

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Abstract

The human hepatoma cell line (Hep G2) has retained the activities of various phase I and phase II enzymes which play a crucial role in the activation/detoxification of genotoxic procarcinogens and reflects the metabolism of such compounds in vivo better than experimental models with metabolically incompetent cells and exogenous activation mixtures. In recent years, methodologies have been developed which enable the detection of genotoxic effects in Hep G2 cells. Appropriate endpoints are the induction of 6-TG^r mutants, of micronuclei and of comets (single cell electrophoresis assays). It has been demonstrated that various classes of environmental carcinogens, such as nitrosamines, aflatoxins, aromatic and heterocyclic amines and polycyclic aromatic hydrocarbons can be detected in genotoxicity assays with Hep G2 cells. Furthermore, it has been shown that these assays can distinguish between structurally related carcinogens and non-carcinogens, and positive results have been obtained with rodent carcinogens (such as safrol and hexamethylphosphoramide) which give false negative results in conventional in vitro assays with rat liver homogenates. Hep G2 cells have also been used in antimutagenicity studies and can identify mechanisms not detected in conventional in vitro systems such as induction of detoxifying enzymes, inactivation of endogenously formed DNA- reactive metabolites and intracellular inhibition of activating enzymes.

Key words: Metabolic activation, human Hep G2 cells, mutagenicity, antimutagens

Introduction

The majority of genotoxicity studies are carried out under *in vitro* conditions with bacterial and mammalian indicator cells which are devoid of enzymes involved in the activation of promutagens. In order to compensate for the lack of drug metabolizing enzyme systems, exogenous activation mixtures (usually S9 enzyme fractions from rodents) are added in these experiments. It is known since many years that the limitations of the predictive value of short term *in vitro* assays are partly due to inadequate representation of the drug metabolism (Bigger *et al.*, 1980; Bos *et al.*, 1983; Ashby, 1980 Tennant *et al.*, 1987) and it is well documented that substantial differences in genotoxicity experiments are obtained when cellular or subcellular hepatic activation systems are used (see for example (Bos *et al.*, 1983;

* *Author for correspondence and reprints;* Abbreviations on last page of the paper.

Utesch, 1987)). Consequently, attempts have been made to develop indicator organisms with an improved endogenous activation capacity (Rueff, 1996). Basically, three different approaches have been developed: (i) Use of primary hepatocyte cultures from rodents and humans (Brambilla and Martelli, 1990; Butterworth *et al.*, 1989; Muller – Teggethoff, 1995; Eckd *et al.*, 1987; Salissidis *et al.*, 1991). Although the cultivation methods have been improved over the years, primary cultures still lose their drug metabolizing capacity rapidly after isolation (Guguen – Guillouzod Guillouzo, 1983; Strom *et al.*, 1982; Michalopoulos *et al.*, 1976) and undergo only a limited number of cell divisions. The use of liver cells from humans is often restricted due to limited availability of bioptic material. (ii) As an alternative, several groups have constructed genetically engineered cells which express certain drug metabolizing phase I and phase II enzymes (Crespi, 1995). These cells have been extremely useful for mechanistic studies, but since only a restricted number of enzymes is expressed in each cell line, their use for routine testing of unknown compounds is of low predictivity. (iii) Finally, permanent cell lines were propagated for genotoxicity studies which have retained endogenous bioactivation capacity. Most of these lines are derived from hepatic tissue since the liver is the most important drug metabolizing organ in mammals. Several groups have worked with rodent liver lines (Roscher and wiebel, 1988; Dean *et al.*, 1980; Dean *et al.*, 1983; Tong *et al.*, 1981; Tong *et al.*, 1984; Salvia *et al.*, 1988) and obtained promising results with representatives of different groups of procarcinogens. It is known that the activation capacities and substrate specificities of drug metabolizing enzymes show strong interspecies variations (Selkirk, 1977; Maslansky and Williams, 1982), therefore human derived cells appear most advantageous for the detection and risk assessment of chemicals which might pose health hazards to man. Two promising cell systems are available; the first is a SV 40 immortalized human liver endothelial cell line (Pfeifer *et al.*, 1993), the second, on which the present presentation will focus, is the human derived hepatoma line Hep G2.

Hep G2 was isolated in 1979 by Aden and his coworkers (Aden *et al.*, 1979) from a primary hepatoblastoma of an 11 year old Argentinean male. The cells have an epithelial like morphology which resembles liver parenchymal cells and synthesize and secrete many of the plasma proteins characteristic for normal human liver cells (Knowles *et al.*, 1980). Hep G2 cells have an aneuploidic karyotype with a modal chromosomal number of 52 and a range of 48-54 chromosomes per cell (Natarajan and Darroudi, 1991) and are routinely cultivated in standard media such as Earle's Minimal Essential medium with Eagle salts (Hall *et al.*, 1991; Chang *et al.*, 1988) or in Dulbeccos Minimal Essential medium (Natarajan and Darroudi) with 10-15 % fetal calf serum. The impact of the cultivation medium on the activities of drug metabolizing enzymes is discussed below. The generation time of Hep G2 cells is substantially longer (20-28 hrs) than that of the rodent cell lines (V-79, CHO) currently used in genetic toxicology (Natarajan and Darroudi, 1991; Schwab, 1997). Hep G2 cells have shown their versatility in a variety of mutagenicity test systems (see below), in cytotoxicity studies with xenobiotics (Hall *et al.*, 1991; Gergel *et al.*, 1995; Neuman *et al.*, 1993; Shear *et al.*, 1995), in studies concerning the mechanisms of viral (hepatitis B) infections (Mangold and Streeck 1993; Gustin *et al.*, 1993; Wu *et al.*, 1991), dioxin receptor effects (Pimental *et al.*, 1993; Wang *et al.*, 1996), and gene expression and transcription (Kardassis *et al.*, 1992; Babajko, 1995; Rust *et al.*, 1996; Miao *et al.*, 1996) to mention only a few.

Drug metabolizing enzymes in Hep G2 cells

As described above, the major advantage of Hep G2 cells for mutagenicity/anti-mutagenicity studies resides in their expression of phase I and phase II drug metabolizing enzymes (Table 1). Several studies with Hep G2 cells give indirect evidence for the activation of promutagens as outlined in more detail below, but investigators have also directly addressed the metabolic capacity of Hep G2 cells and found substrate specificities similar to those of original human hepatocytes (Table 1).

TABLE 1: Drug metabolizing enzymes in human Hep G2 cells

Enzyme	*Substrate metabolized*	*Remarks*	*Reference*
Phase I enzymes			
Total Cyt. P-450	B(a)P, 7-ethoxycoumarin	2.5 pmol/mg	Sassa *et al.*, (1987)
CYP 1A1/1A2	B(a)P		Diamond *et al.*, (1980)
CYP 1A	B(a)P, aldrin		Limbosch (1983)
CYP 1A1/1A2	B(a)P		Eddy *et al.*, (1987)
CYP 1A1/1A2	ethoxyresorufin	lower activity than human hepatocytes	Grant *et al.*, (1988)
CYP 1A, CYP 2B	substituted resorufins	optimal activity by medium selection	Doostdar *et al.*, (1988)
CYP 1A1, 2C, 3A	acetanilide, phenacetin		Fardel *et al.*, (1992)
CYP 2E1, CYP 1A	acetaminophen, ethoxyresorufin		Roe *et al.*, (1993)
AHH	B(a)P	activity in supernatant only after	Duverger van Bogaert *et al.*, (1993)
	aniline, ethylmorphine	induction, no activity in Hep G2 homogenates	Sassa *et al.*, (1987)
	7,12-DMBA	production of water soluble metabolites	DiGioranni *et al.*, (1984)
Nitroreductase	1-NP		Eddy *et al.*, (1987)
N-Demethylase	aminopyrine	activity in supernatant not detectable	Duverger van Bogaert *et al.*, (1993)
Catalase		activity in supernatant	Duverger van Bogaert *et al.*, (1993)
Peroxidasee	purpurogallin	activity in supernatant	Duverger van Bogaert *et al.*, (1993)
FMO	thiobenzamide	activity in supernatant	Duverger van Bogaert *et al.*, (1993)
NAD(P)H:Cyt c red.		activity in supernatant	Duverger van Bogaert *et al.*, (1993)
Cyt. P450 reductase		similar activity as in human hepatocytes	Grant *et al.*, (1988)
NAD(P)H: Quinone oxidoreductase	menadione, 2,6-dichloro-phenolindophenol	two isoenzymes expressed	Jaiswal *et al.*, (1990)
Phase II enzymes			
Epoxide hydrolase	B(a)P-4,5-oxide		Dearfield *et al.*, (1983)
Sulfotransferase ST	7,12-DMBA	very low sulfo-transferase activity	DiGioranni *et al.*, (1984)
ST	phenol, dopa, tyrosine	pH opt. at 8.75; 27% of max. activity at pH 7.4	Suiko *et al.*, (1996)
ST	l-tyrosine		Sakakibara *et al.*, (1994)

(Table 1 Contd...)

(Table 1 Contd...)

Enzyme	*Substrate metabolized*	*Remarks*	*Reference*
ST		low activity	Fardel *et al.*, (1992)
ST, M-form	propanol, terbutalin, salbutamol	4-fold higher than in human liver	Walle *et al.*, (1994)
ST, P-form	phenol	35% activity of human liver	Walle *et al.*, (1994)
GST	1-chloro-2,4-dinitrobenzene	activity similar to freshly isolated human hepatocytes, mainly alpha class isoenzyme	Doostdar *et al.*, (1988)
GST	2,4-dinitrophenol		Duverger van Bogaert *et al.*, (1993)
UDPGT	7,12-DMBA	very low sulfotransferase activity	DiGioranni *et al.*, (1984)
UDGPT, GST, EH,ST	B(a)P	activity present	Eddy *et al.*, (1987)
UDPGT	phenolphthalein, 1-naphthol	similar levels as in human hepatocytes	Grant *et al.*, (1988)
UDPGT	1-naphthol, bilirubin	optimal activity by medium selection	Doostdar *et al.*, (1988)
N-acetyltransferase	p-aminobenzoic acid; anisidine		Coroneos and Sim (1993)
NAT		low levels	Fardel *et al.*, (1992)

However, systematic comparisons of both activation systems are still lacking. In any event, enzyme activities are due to variation by the culture conditions in both primary cultures of human hepatocytes and in Hep G2 cells. The influence of medium composition in the latter was studied by Doostdar *et al.*, (1988), who found that modified Earle's medium was superior to two other media in maintaining high phase I enzyme activities, whereas phase II enzymes were less affected. Only the glutathione levels were reported to be increased under these conditions.

Isoenzymes of several CYP families are present in Hep G2 which are responsible for the activation of diverse promutagens, e.g. CYP 1A for PAHs and aromatic amines, CYP 2E1 for nitrosamines, CYP 2B for several chemically unrelated drugs, and CYP 3A for nitropyrenes and aflatoxins. Apart from the cytochrome P450 isoenzymes, several other oxidoreductases involved in either electron transfer processes to cytochrome P-450 or in the activation of reactive oxygen species have been detected in Hep G2 cells (see Table 1). The presence of epoxide hydrolase activity [63] enables the formation of dihydrodiols from PAH epoxides which then can be further activated by CYP 1A enzymes to the respective dihydrodiolepoxides as the ultimate mutagens/carcinogens.

All of the known phase II enzymes tested so far were shown to be present in Hep G2 cells (see lower part of Table 1). Their activities varied with the different substrates from very low (UDPGT, ST), to similar as in freshly isolated human hepatocytes (UDPGT, GST), and even to higher than in human hepatocytes (ST, M-form). In some cases, optimal activity might be acquired by selection of the appropriate medium conditions (Doostdar *et al.*, 1988). The presence of conjugating enzymes is of dual importance: First, compounds undergoing metabolic activation following conjugation, like the vicinal haloethanes

by GST might be detected in mutagenicity tests; second, the probability that ultimate mutagens will be damaging DNA is reduced by subsequent conjugation reactions. Thus, the Hep G2 cell system can be expected not to be over sensitive but rather reflecting the metabolic status similar to human liver cells. Since Hep G2 cells were derived from a human hepatoblastoma, it is anticipated that the overall drug metabolizing enzymatic activity will be lower than in normal liver (Cameron *et al.*, 1976; Sultanos and Vesell, 1980).

The lower constitutive enzymatic activities in the tumor cells may be enhanced by a variety of known enzyme inducers, e.g. induction was reported for phase I enzymes CYP 1A by aroclor (Babich *et al.*, 1988), TCDD (Labruzzo *et al.*, 1989), 1,2-benzanthracene (BA, (Grant, 1988; Fardel, 1992)) and 3-methylcholanthrene (3MC, (Belisario *et al.*, 1991)); CYP 2B by phenobarbital (PB) and BA (Grant, 1988; Fardel, 1992); CYP 2E1 by acetone (Roe *et al.*, 1993); CYP 3A by dexamethasone (Fardel *et al.*, 1992) and rifampicin (Fardel *et al.*, 1992; Doo stdar *et al.*, 1993). Phase

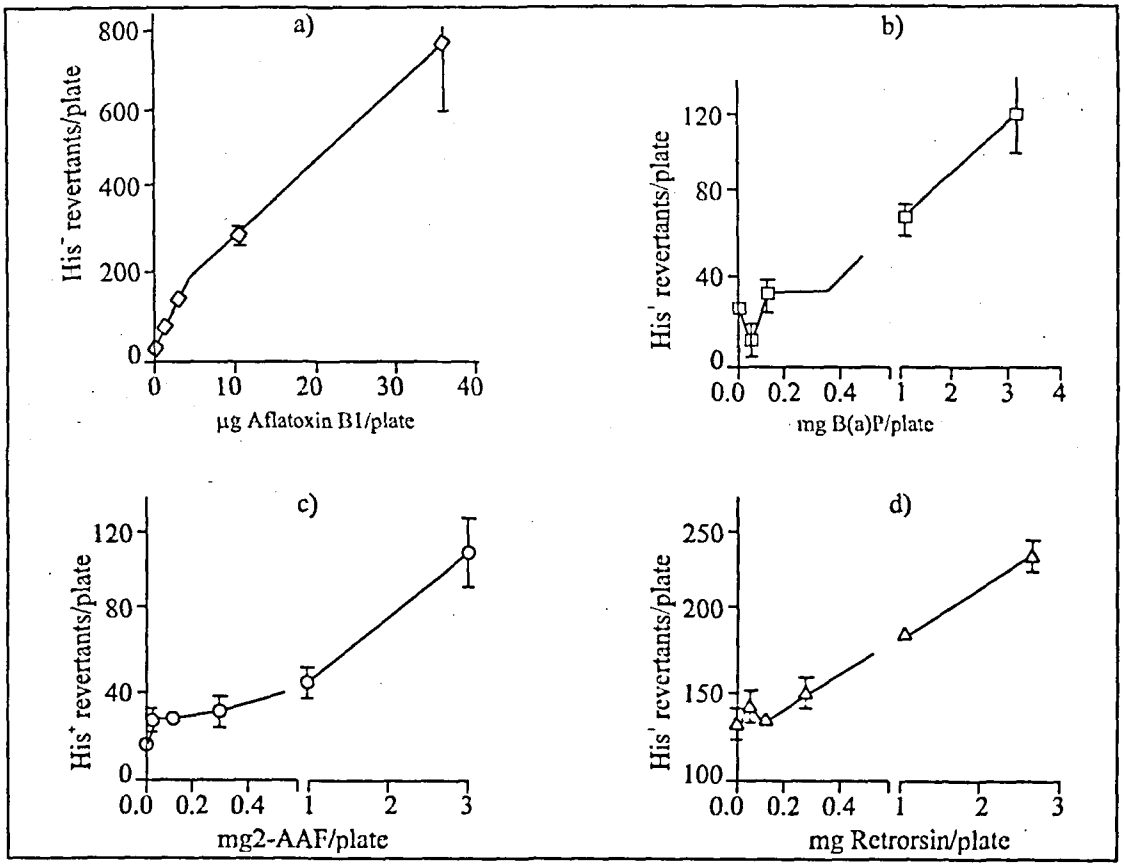

FIGURE 1: *Induction of HIS⁺ revertants by representatives of different classes of promutagens in Salmonella typhimurium tester strains with Hep G2/S9 mix. The test compounds B(a)P, AFB_1 and 2-AF were dissolved in DMSO and tested with Hep G2/S9 mix (composed according to Darroudi and Natarajan, 1994) in plate incorporation assays. Retrorsine was tested with the preincubation method Maron and Ames, 1983. Per experimental point three plates were evaluated.*

II enzymes were induced as follows: UDPGT by PB, BA, dexamethasone, rifampicin, and 3MC (Grant *et al.*, 1988; Doostdar *et al.*, 1993; Duthie *et al.*, 1988); N-acetyltransferase by dexamethasone, rifampicin and 3MC (Fardel *et al.*, 1992) and GST by extracts from vegetables (Du Pont *et al.*, 1994).

Use of Hep G2 cells in mutagenicity tests

Shortly after the isolation of the cell line, Diamond and her coworkers (Diamond *et al.*, 1980) reported that Hep G2 cells are capable to convert B(a)P to mutagenic intermediates as indicated by a positive result in an experiment, where Hep G2 cells were cocultivated with V-79 cells (endpoint $6TG^r$).

The numerous mutagenicity experiments which have been carried out with Hep G2 cells can be grouped into three methodological categories.

Studies with enzyme homogenates

In the first category, enzyme fractions were prepared from the hepatoma cells and used in combination with bacterial or mammalian indicator cells. Duverger *et al.*, (1993, 1995) obtained positive results with aromatic amines and PAHs in a modified version of the Ames test where the *Salmonella* TA98 strain was pretreated with a Hep G2 derived S2 supernatant. A pronounced increase of the mutagenic response was measured when the cells were preincubated with the enzyme inducers hydrocortisone and BA, and the mutagenicity of the aromatic amines was elevated in TA98 derivatives which express enhanced levels of acetyltransferase. We have used the same protocol in experiments with HAAs: with the parent strain (TA98) only marginal mutagenic effects were detectable whereas with strain YG 1024, which has an elevated O-acetyltransferase activity (Watanabe *et al.*, 1990), pronounced effects were measured (Schwab, 1997). The mutagenic potencies of different amino acid pyrolysates was distinctively different from that seen in standard Ames tests with TA98 and rat S9 mix (Hep G2/S2 activation: IQ ³ MeIQ > Trp-P-1 > MeIQx > PhIP; rat liver/S9 activation: MeIQ > IQ > MeIQx > Trp-P-1 > PhIP). Darroudi and Natarajan (1993) developed a recipe for a Hep G2 S9 mix which we used in a series of plate incorporation experiments with *Salmonella* strains TA98 and TA100. Some of the results are depicted in Figures 1 a-d.

The strongest effect was seen in TA 98 with the mycotoxin AFB_1, whereas B(a)P, 2-AF and the pyrolizidine alkaloid retrorsine induced only moderate effects at relatively high dose levels in strain TA100. With NDMA, a clear negative result was obtained under all conditions of test up to 3 mg/plate (data not shown). Note that the nitrosoamine is also negative in standard Ames tests with rat liver S9 activation (Yahagi *et al.*, 1977; Rao *et al.*, 1979). Darroudi and coworkers (1993, 1994, 1996) used the Hep G2/S9 mix in experiments with CHO cells: endpoints were induction of MN, SCEs, gene mutations ($6\text{-}TG^r$) and cell survival. Positive results were obtained with standard mutagens such as B(a)P and CP. More interestingly, two problematic substances, safrol and HMPA, which are rodent carcinogens but give false negative results in other *in vitro* assays, caused clear cut positive responses. Pyrene and 4-AAF which are not carcinogenic in rodents but structurally closely related to carcinogens were devoid of mutagenic activity in this experimental model. We have recently tested five HAAs in the CHO/Hep G2/S9 system (endpoints $6\text{-}TG^r$ and MN induction): PhIP and Trp-P-1 caused positive results (dose range 0.01-0.4 mM, exposure time 2 hrs), whereas quinoline and quinoxaline compounds (IQ, MeIQ, MeIQx) were not mutagenic (Schwab, 1997).

Cell mediated assays

The second group of experiments are "cell mediated assays" in which intact Hep G2 cells are cocultivated with metabolically incompetent indicator cells together with the test compounds. Zhou *et al.*, (Zhou *et al.*, 1986) tested some representatives of promutagens with bacterial indicators (*Salmonella* TA98) and obtained positive results with 2-AF, 2-AA, benzidine and 4-aminobenzo(a)pyrene, whereas no activity was found with B(a)P, DMBA, 4-AB and 11-aminobenzo(a)pyrene. The exposure concentrations

TABLE 2: Induction tests with Hep G2 cells

Test comp.[1]	*Method*	*Exp. (h)*	*Dose range*	*m.e.d.*	*Unit*	*Result*[2]	*Remarks*	*Ref.*[3]
Detection of single strand breaks								
B(a)P	SCGE	24	15	(15)	µM	+	Frozen cells with low viability	Rompelberg *et al.*, (1996)
	SCGE	4	200	(200)	µM	+	Viable cells optimized method	pc.
Eugenol	SCGE	24	0.003-1.0		µM	-	Frozen cells with low viability	Rompelberg *et al.*, (1996)
DADS	SCGE	4	5		µM	-	Viable cells, optimized method	pc.
IQ	SCGE	24	800		µg/ml	+		
Nitrosamines								
NDMA	AE	20	50-100	(50)	mM	+	in addition cytotox. tests and	Brambilla-Campart *et al.*, (1989); Gardina *et al.*, (1992)
NDEA	AE	20	18-56	(56)	mM	+	Comparisons with the effects	
NDPA	AE	20	10-32	(18)	mM	+	in primary human hepatocytes,	
NDBA	AE	20	1.0	(1.0)	mM	+	Different ranking order	
NMOR	AE	20	1.8-5.6	(5.6)	mM	+	of genotoxic potency, primary	
NPIP	AE	20	1.8-5.6	(5.6)	mM	+	Human cells more sensitive	
NPYR	AE	20	18-50	(50)	mM	+		
Nitrosureas								
NEU	AE	20	1.0-5.6	(1.0)	mM	+	Comparison with the effects in	Gardina *et al.*, (1992)
NMU	AE	20	1.0-5.6	(1.0)	mM	+	Primary human hepatocytes	
NBU	AE	20	1.0-3.2	(3.2)	mM	+		
4-NQO	AUA	24	0.25-1.0	(0.2)	µM	+	new method	Hasspieler *et al.*, (1995)
Phenanthrene-9,10-quinone	AUA	24	0.25-0.75	(0.5)	µM	+	new method	Hasspieler *et al.*, (1995)
Unscheduled DNA Synthesis								
B(a)P		3	1-1000	(7.5)	µM	+	Stronger effect as in rat liver cells	Liu *et al.*,
MMS		24	10-50	(20)	µM	+		Naji-Ali *et al.*, (1994)
MNNG		3	13	(13)	µM	+		Eddy *et al.*, (1987)
Nitroarenes								
1-NP		3	0.4-8.2	(2.0)	µM	+		Eddy *et al.*, (1987)
		3	4	(4.0)	µM	+		Silvers *et al.*, (1994)
1,3 DNP		3	1.7-6.8		µM	-	Negative results obtained with	

(Table 2 Contd...)

(Table 2 Contd...)

Test comp.[1]	*Method*	*Exp. (h)*	*Dose range*	*m.e.d.*	*Unit*	*Result*[2]	*Remarks*	*Ref.*[3]
1.6 DNP		3	1.7-6.8		µM	-	Dinitropyrenes are probably	
1.8 DNP		3	1.7-6.8		µM	-	due to lack of O-esterificase	
4-NQO		24	0.2-1.0	(0.2)	µM	+		Naji-Ali *et al.*, (1994
		3	2.7-5.3	(5.3)	µM	+		Eddy *et al.*, (1987)
Sister Chromatid Exchanges								
B(a)P		1	0.01-0.08	(0.04)	mM	+		Natarajan and
		28	0.01-0.02	(0.01)	mM	+		Farroudi (1991)
Benzidine		72	0.1-10	(5.0)	µg/ml	+	Similar effect in rat hepatoma cells	Grady *et al.*, (1986)
Benznidazole		1	25-100	(25)	µg/ml	+	Antitrypanosomal drug	Santos *et al.*, (19994)
CP		72	25-1000	(50)	µg/ml	+	Similar effects in rat hepatoma cells	Dearfield *et al.*, (1986)
HMPA		1	3-18	(9)	µM	±	Rodent carcinogen, negative in other *in vitro* tests	Natarajan and Farroudi (1991)
MMC		1	0.25-1.00	(0.5)	mM	+		Natarajan and Farroudi (1991)
MMS		1	0.4-1.20	(0.8)	mM	+		Natarajan and Farroudi (1991)
NDM		1	10-50	(50)	mM	+		
		28	5-30	(20)	mM	+		
Phosphamide-mustard		72	0.025-1.0	(0.05)	µg/ml	+	Direct active metabolite of CP	Dearfield *et al.*, (1986)
Pyrene		1	0.04-0.16		mM	-	not carcinogenic in rodents,	Natarajan and Farroudi (1991)
		28	0.04-0.16		mM	-	pos. in other *in vitro* tests	
Safrol		1	0.1-0.3	(0.1)	mM	±	Carcinogenic in rodents,	Natarajan and Farroudi (1991)
		28	0.1-0.15	(0.15)	mM	±	Negative in other *in vitro* tests	

[1] SCGE-single cell gel electrophoresis assay, AE-alkaline eluation, AUA-alkaline unwinding assay, [2] m.e.d-minimal effective dose (in the reported experiment), + positive result, ± marginal effect, - negative result [3] pc.-present communication

TABLE 3: Gene mutation tests with Hep G2 cells[1)]

Test compound	*Dose range*	*m.e.d.*[3)]	*Unit*	*Result*[2)]	*Remarks*	*Reference*
AFB_1	0.2-2	(0.50)	µg/ml	+	use of feeder cells	Chang *et al.*, (1998)
AZT (1992)	0.25-5	(0.25)	g/ml	+	anti aids drug, negative in 6-TG tests with CHO cells	Gardina *et al.*,
CP	1.0-10	(7.5)	mg/ml	+	use of feeder cells	Chang *et al.*, (1998)
MNNG	6.8	(6.8)	µM	+		Eddy *et al.*, (1987)
Nitroarenes						
1-NP	10	(10)	µM	+		Silvers *et al.*, (1994)
	2-20	(4.0)	µM	+		Eddy *et al.*, (1987)
1,3-DNP	3.4-17		µM	-	negative results probably	Silvers *et al.*, (1994)
1,6-DNP	3.4-17		µM	-	due to lack of	
1,8-DNP	3.4-17		µM	-	O-esterificase	
4-NQO	5.3	(5.3)	µM	+		Eddy *et al.*, (1987)
UV	5-25	(5.0)	J/m^2	+	254 nm, use of feeder layer	Chang *et al.*, (1998)

[1)] Endpoint: 6TG-resistance, exposure time: 3hrs; [2)] + positive result, ± marginal effect, - negative result; [3)] m.e.d.-minimal effective dose

in these experiments were low (£ 50 µg/ml) and per compound only two concentrations were tested. The results of Lindahl-Kissling *et al.*, [80] with cyclopenta(a)phenanthrenes (CPPs) in SCE assays with human lymphocytes as indicator cells were more consistent with the *in vivo* findings: The rodent carcinogen 15,16-dihydro-11-methyl-CCP-17-one caused a strong positive effect

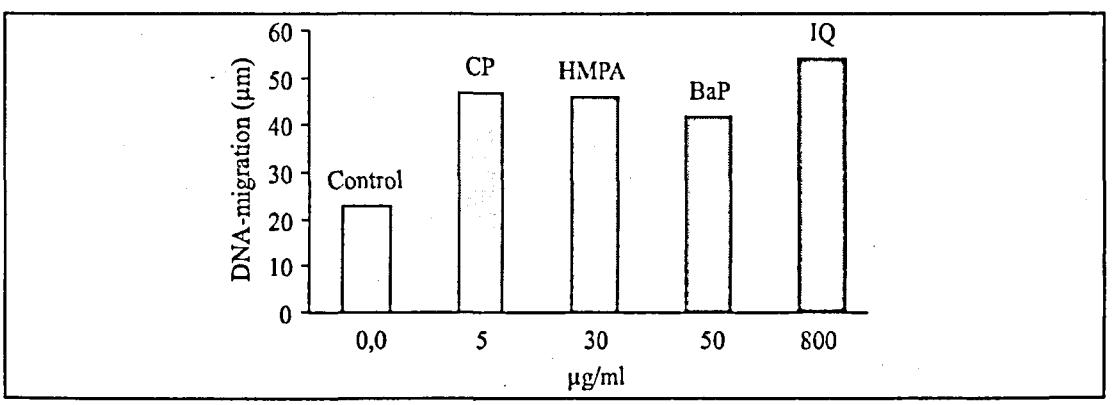

FIGURE 2: Results of SCGE assays with Hep G2 cells. The cells were grown in DMEM with 15% fetal calf serum, trypsinized and exposed to different concentrations of the test compounds for 4 hrs. Subsequently, the cells were lysed (2,5M NaCl, 100 mM EDTA, 10 mM Tris, 1% TritonX100, pH 10) in presence of 10% DMSO Roscher and Wiebel (1992) for 1 hr, subjected to alkaline treatment (pH > 13.1; 20 min), electrophoresed (20 min, 300 mA, 25 mV) and stained with ethidium bromide (20 µg/ml). Per experimental point the means of the total tail lengths of the comets of 150 cells were determined with a semiautomatic image analyzing system (NIH- Image).

TABLE 4: Micronucleus assays with Hep G2 cells

Test compound	*Exposure (hrs)*	*Exposure range*	*Dose*	*m.e.d.*	*Unit*	*Res.*[1]	*Remarks*[2]	*Ref.*[3]
2-AAF	19	0.1-0.4	(0.1)	µM	+		FISH:C+30/C-70, rodent carcinogen	Darroudi *et al.*, (1996)
4-AAF	19	0.4-0.8		µM	-		not carcinogenic in rodents	Darroudi *et al.*, (1996)
B(a)P	1	.01-0.16	(0.08)	mM	+			Natarajan and Farroudi (1991)
	28	0.01-0.16	(0.16)	mM	+			Natarajan and Farroudi (1991)
	4	25-600	(25)	µM	+			Mohn *et al.*, (1997)
Benznidazole	1	25-200	(200)	g/ml	±		positive in SCE tests	Salvadori *et al.*, (1993
Caffeine	24	5-500	(500)	µg/ml	+		positive in other *in vitro* tests	Santos *et al.*, (1994)
Chrysin	4	2.5-35	(15)	µg/ml	+		negative in bacterial tests	Mohn *et al.*, (1997)
Cinnamaldehyde	24	5-500	(500)	µg/ml	+		bioantimutagen in bacteria	Santos *et al.*, (1994)
Coumarin	24	5-500	(500)	µg/ml	+		positive in other *in vitro* tests	Santos *et al.*, (19994)
CP	24	10^{-8}-10^{-2}	(10^{-2})	M	+			Natarajan and Farroudi (1991)
	19	2.5-10	(2.5)	mM	+		FISH:C+14/C-86	Darroudi *et al.*, (1996)
Diallyldisulfide	24	5		µM	-		pc.	
HAAs								
IQ	24	0.025-0.9	(0.05)	mM	+		different ranking order as *in bacterial*	[34]
	4	0.6	(0.6)	mM	+		mutagenicity tests, better correlation of	Sanyal *et al.*, (1997)
MeIQ	24	0.025-0.9	(0.05)	mM	+		the ranking order of relative genotoxic	[34]
	4	0.9	(0.9)	mM	+		activities with the carcinogenic	Sanyal *et al.*, (1997)
MeIQx	24	0.025-0.9	(0.05)	mM	+		potencies in rodents as with data from	[34]
	4	0.9	(0.9)	mM	+		bacterial *in vitro* tests or MN data	Sanyal *et al.*, (1997)
Trp-P-1	24	0.23-25	(12.5)	µM	+		obtained with metabolically	[34]
	4	0.012	(0.012)	mM	+		incompetent cell lines.	Sanyal *et al.*, (1997)
PhIP	24	0.025-0.9	(0.05)	mM	+			[34]
	4	0.6	(0.6)	mM	+			
HMPA	1	3-27	(18)	µM	+		rodent carcinogen, negative in other	Natarajan and Farroudi (1991)
	28	3-26	(9)	µM	+		*in vitro* tests	Natarajan and Farroudi (1991)
	19	2.5-10	(2.5)	µM	+		FISH:C+/C-	Darroudi *et al.*, (1996)
MMS	1	0.8-1.6	(1.6)	mM	±			Natarajan and Farroudi (1991)
	28	0.8-1.6	(0.8)	mM	+			
	19	0.1-0.3	(0.1)	mM	+		FISH:C+30/C-70	Darroudi *et al.*, (1996)

(Table 4 Contd...)

(Table 4 Contd...)

Test compound (hrs)	*Exposure range*	*Dose*	*m.e.d.*	*Unit*	*Res.*[1)]	*Remarks*[2)]	*Ref.*[3)]
MMC (1991)	1	0.25-1.0	(0.9)	µM	+		Natarajan and Farroudi
	1	1	(1)	µM	+		Salvadori *et al.*, (1993)
Musk ketone	48	0.005-5		µg/ml	-		pc.
Nitroarenes (1992)							Roscher and Wiebel
1,3-DNP	24	3.0	-	µM		positive results in other mammalian	
1,6-DNP	24	3.0		µM	-	cell lines	
NDMA	1	0.5-60	(60)	mM	+	negative in standard Ames Test	Natarajan and Farroudi (1991)
	28	0.5-30	(15)	mM	+	negative in bone marrow MN	
Pyrene	1	.08-0.32			-	negative in rodent carcinogenicity	Natarajan and Farroudi (1991)
	28	.08-0.32		mM	-	tests, positive in *in vitro* tests	
Safrol	1	.07-1.20	(1.2)	mM	+	rodent carcinogen, negative in	Natarajan and Farroudi (1991)
	28	.07-1.20	(1.2)	mM	+	most *in vitro* tests	
Tannic acid	24	5-500	(500)	µg/ml	+	bioantimutagen in bacteria	Sanyal *et al.*, (1997)
Vanillin	24	5-500	(500)	µg/ml	+	bioantimutagen in bacteria	Sanyal *et al.*, (1997)
Vinblastine	19	.01-0.10	(0.01)	µM	+	aneugen, FISH:C+87/C-13	Darroudi *et al.*, (1996)
X-rays	n.s.	1-3	(1)	Gy	+	FISH:C+35/C-64	Darroudi *et al.*, (1996)

[1)] m.e.d.-minimal effective dose (in the reported experiment), + positive result, ± marginal effect, - negative result; [2)] FISH - fluorescence *in situ* hybridization, C+ % centromeric probe positive MN, C- % centromeric probe negative MN, ns.- not specified; [3)] pc.-present communication

whereas the unmethylated parent compound, which is classified as non carcinogenic, gave a negative result. The latter compound causes false positive results in HIS$^+$ reversion assays with *Salmonella* and in 6-TG^r tests with V-79 cells. The carcinogenic methyl derivative was negative in cocultivation tests with V-79 cells and low passage hamster embryo cells (endpoint 6-TG^r), but a positive result was obtained in the same model with Hep G2 cells (Bhatt etla, 1983). In addition, a series of other CPP derivatives were assayed in Hep G2/V-79 6-TG^r tests and in general a good correlation of the relative mutagenic potencies of these compounds with their carcinogenic activities in the mouse skin model was noted. Also methylated and fluoro-substituted derivatives of PHAs (benzo(a)pyrenes, methycholanthrene and dibenzo(a,h)-anthracenes) caused positive results in this model and again the relative mutagenic potencies correlated well with the skin tumor initiating activities in SENCAR mice (Di Giovanni *et al.*, 1984; Diamond *et al.*, 1984).

Detection of genotoxic effects in Hep G2 cells

In both aforementioned models, reactive intermediates are formed outside the indicator cells and metabolites of large molecular size, with high reactivity or short half lives may not reach the DNA in the target cells. These limitations can be avoided by directly measuring genotoxic effects in the activating cells. The results of such studies are summarized in Tables 2-4.

The most frequently used methods are the alkaline elution technique, determination of the induction

of SCEs, MN or 6TG^r mutations. Rompelberg *et al.* (1996) recently reported results from SCGE experiments with B(a)P and eugenol. Their assay procedure was not adapted specifically for Hep G2 cells and the incubation of deep frozen cells with a low viability may need caution in the interpretation of their results. We have recently developed an improved protocol for SCGE experiments and obtained positive results with different promutagens (Fig. 2).

Another useful approach is the MN test which is time and cost effective. Since the first report on MN induction in Hep G2 cells (Natarajan and Darroudi, 1981), the data base has increased substantially (Table 4). Recently, improved techniques with centromeric probes have been developed which enable the discrimination between aneugenic and clastogenic effects (Darrudi *et al.*, 1996).

In general, the results of these experiments indicate that the effects of representatives of various important classes of promutagens/procarcinogens such as nitrosamines, PAHs, and aromatic and heterocyclic amines can be detected in genotoxicity studies with Hep G2 cells. In addition, rodent carcinogens such as safrol and HMPA can be detected which usually give false negative results in other *in vitro* assays. The only class of rodent carcinogens which consistently gave negative results in Hep G2 assays are dinitropyrenes (Eddy *et al.*, 1987; Silvers *et al.*, 1994; Roscher and Wiebel, 1992), the reason for the negative effect is probably the absence of activation by O-esterificase (Silves *et al.*, 1994). It is known that the carcinogenic effects of dinitropyrenes are depending on species, route of administration and administration of relatively high doses (IARC, 1989). Therefore it is questionable if these compounds indeed pose a cancer risk for humans. HAAs caused clear cut positive results in MN assays with Hep G2 cells. The ranking order of mutagenic potencies established on the basis of these experiments is different from that seen in bacterial mutagenicity tests and correlates much better with their carcinogenic activities in rodents (Schwab, 1997). On the contrary, negative results were obtained with quinolines and quinoxalines in experiments with CHO cells (see above). It is known that these later compounds require activation by acetyltransferases (Wild *et al.*, 1995; Yanagawa *et al.*, 1994). We have shown that Hep G2 cells express substantially higher N-acetyltransferase activities than CHO cells (Schwab 1997; Schwab, 1996). It is notable in this context that strong rodent carcinogens such as IQ give also false negative results in the mouse bone marrow MN tests (Wild *et al.*, 1985; Loprieno *et al.*, 1991). Similarly, no clear positive result was obtained in MN assays *in vivo* with PhIP, which is tumorigenic in mice and rats (Tucker *et al.*, 1987).

Use of human hepatoma cells for the detection of antimutagens

About 40 years ago, the first antimutagens have been detected (for survey see Gebhart, (Gebhart, (1974)) and since then numerous studies have been carried out in order to identify compounds which might protect humans against DNA-damage and its consequences. The basic assumption over the years has been that any test system which can be used for the detection of mutagens would be appropriate for the detection of antimutagens as well (Gebhart, (1974). So far, the vast majority of antimutagenicity studies have been performed under *in vitro* conditions, in particular with bacterial indicators and it has been proposed that antimutagens identified in cost and time effective *in vitro* experiments with exogenous metabolic activation systems should be further evaluated in animal studies (Ferguson, 1994). On the other hand, biochemical studies have elucidated the mechanisms which are responsible for the mode of action of antimutagens (De Flora and Ramel, 1988) and it became apparent that mechanisms such as induction of detoxifying enzymes cannot be detected in assays with metabolically incompetent cells. Furthermore, it is possible that putative antimutagens interact with enzymes in exogenous activation mixtures to give incorrect results. A typical example for such a case is ethanol which inhibits the effects of nitrosamines in bacterial *in vitro* tests whereas it enhances the

TABLE 5: Antimutagenicity studies with Hep G2 cells

Putative antimutagen	*Endpoint*	*Dose range*		*Mutagen/dose*			*Treatment*[1]	*Result*	*Remarks m.e.d.*[2]	*Ref.*[3]
Compounds which interact with metabolic activation or reactive metabolites										
ß-Carotene	MN	0.25-6.0	µM	CPA	400	µM	pre	↓ (0.25)	probably interaction with metabolic activation	Salvadori *et al.*, (1993)
				MMC	1	µM	pre+sim	↓ (0.25)		
							sim	↓ (0.25)		
							sim	-		
Chrysin	MN	1.3-33	µg/ml	B(a)P	0.6	mM	pre+sim	↓ (3)	inact. BPDE, enzyme inhibit.	Mohn *et al.*, (1997)
	MN	1.3-33	µg/ml	PhIP	0.6	mM	pre+sim	↓ (4.4)		
Diallyldisulfide	SCGE/MN	5	µM	B(a)P	50	µg/ml	pre	↓ (5)	induction of GST pc.	
Eugenol (1996)	SCGE	3-100	µM	B(a)P	15	µM	pre	↑ (10)	*	Rompelberg *et al.*,
							sim	↑ (10)		
							post	-		
Indomethacin	SCE	1	mM	benzi-dine	0.1-10	µg/ml	pre+sim	↓ (1)	inhibit. of prosta-glandine syntethase	Grady *et al.*, (1986)
Metyrapone (1986)	SCE	10-1000	µM	CP	1.9	mM	pre	↓ (10)	P-450 inhibition	Dearfield *et al.*,
Musk ketone	MN	1	µg/ml	B(a)P	0.2	µg/ml	pre	↑ (1)	induction of IA1 pc.	
Phenetylisothio-cyanate (1996)	MN	0.25-1	µg/ml	NDMA	2	mg/ml	pre+sim	↓ (0.5)	inhibition of a-C-hydroxylation	Knasmüller *et al.*,
	MN	0.25-1	µg/ml	PhIP	120	mg/ml	pre+sim	-	antimutagenic in bacterial tests	Knasmüller *et al.*, (1996)
Bioantimutagens										
W-151326	6-TG[r]	4	mM	AZT	5	mg/ml	sim.	↓ (4)	pres. interaction with DNA-rep.	Gardina *et al.*, (1992)
Aminothiol						-	post	↓ (4)		
Caffeine	MN	1-500	µg/ml	IQ	120	µg/ml	post	↓ (1)	caffeine, vanilline	Sanyal *et al.*, (1997)
Coumarin	MN	1-500	µg/ml	IQ	120	µg/ml	post	↓ (1)	and coumarine inhibited also the	Sanyal *et al.*, (1997)
Cinnamaldehyde	MN	5-500	µg/ml	IQ	120	µg/ml	post	↓ (5)	effects of other	Sanyal *et al.*, (1997)
Tannic acid	MN	5-500	µg/ml	IQ	120	µg/ml	post	↓ (5)	HAAs (MeIQ, MeIQx,	Sanyal *et al.*, (1997)
Vanillin	MN	1-500	µg/ml	IQ	120	µg/ml	post	↓ (5)	Trp-P-1 and PhIP)	Sanyal *et al.*, (1997)

[1] pre - pretreatment (antimutagen exposure before exposure to the mutagen); pre+sim - pretreatment and simultaneous treatment; sim - simultaneous treatment; post - posttreatment; * data can not be interpreted, methodological insufficiences; [2] m.e.d. -minimal effective dose of the putative antimutagen; ↓ decrease (antimutagenic effect); ↑ increase (synergistic effect); - no effect; [3] p.c.-present communication,

mutagenic effects of nitrosamines *in vivo via* induction of cytochrome P-450 IIE1 (Knasmuller *et al.*, 1994).

It is not known at present, if antimutagens which inactivate DNA-reactive intermediates formed in conventional activation mixtures will also detoxify endogenously generated metabolites, and if antimutagens, which act on the level of DNA-repair in bacteria, are effective in mammalian cells as well.

In order to elucidate whether or not mutagenicity tests with Hep G2 cells might provide a more appropriate approach for the detection of antimutagens than conventional *in vitro* tests, we carried out a number of experiments. The results of these studies are summarized together with data created by other groups in Table 5.

To clarify, if bioantimutagens which reduce mutagenic effects in bacterial indicators *via* interaction with DNA-repair and/or replication processes (Ohta, 1993; Kada *et al.*, 1986) are effective in the human derived cells, we tested a panel of food constituents (vanillin, coumarin, cinnamaldehyde, caffeine and tannic acid) in combination with HAAs. Three of these compounds (vanillin, caffeine and coumarin) inhibited the induction of MN in Hep G2 cells by IQ and other HAAs at extremely low dose levels (≤ 5 µg/ml), but at elevated exposure concentrations (≥ 500 µg/ml) all of the compounds caused an induction of the MN frequencies by themselves (Sanyal *et al.*, 1997).

The antimutagenic effects of chrysin and of other bioflavonoids towards B(a)P in *Salmonellal* microsome assays are at least partly due to inactivation of the diol epoxide, but it is not known if inactivation also takes place when the metabolite is formed within mammalian cells. Our experiments with Hep G2 cells indicate that chrysin indeed reduces the induction of MN by B(a)P at low exposure concentrations (Mohn *et al.*,

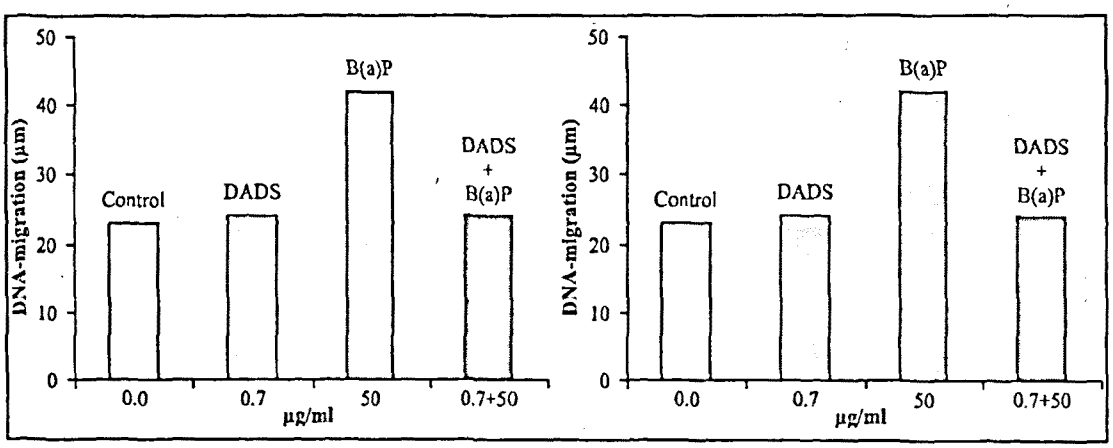

FIGURE 3: *Effect of diallyldisulfide (DADS) on B(a)P induced Mnand comet formation in Hep G2 cells. The cells were exposed for 24 hrs to DADS in serum free medium, subsequently the GST activities were measured sprectrophotometrically using 1-chloro-2,4-dinitrobenzene as a substrate (Gudi and Singh, 1991). In parallel, DADS exposed cells were grown for 3 hrs in medium which contained B(a)P (50 µg/ml medium). After addition of cytochalasin for one cells cycle, the induction of MN was determined as described (Natarajan and Darroudi, 1981) (Fig. 3a). In another parallel series, DADS and B(a)P treated cells (see above) were analyzed in SCGE tests (see Fig. 3b) as described in the legend of Figure 2. In MN experiments 2000 cells were evaluated per experimental point, in the SCGE tests the tail moments of 150 cells were measured per point.*

1997). The dose response curve is U-shaped and further tests showed that the increase of the MN frequencies at elevated chrysin concentration (≥ 30 µg/ml) is due to genotoxic properties of the flavonoid which are not detected in bacterial tests (Mohn *et al.*, 1997).

It has been demonstrated in several animal studies that one of the most important mechanisms for cancer chemoprevention by dietary constituents is the induction of GSTs which inactivate DNA-reactive electrophiles (Sparnins *et al.*, 19821 Watternberg and Lam, 1981). This mode of action cannot be detected in conventional *in vitro* assays (Wiebel, 1993) but spectrometric measurements indicated that incubation of Hep G2 cells with vegetable juices leads to an increase of their GST activity (Du pont *et al.*, 1994). The most pronounced effects (enhancement 1.4-1.6 fold over the background level) were seen with juices of onions and *Brassica* species and it is known that these vegetables contain GST inducing constituents (Du pont *et al.*, 1994). The mode of action of GST induction in Hep G2 cells is unclear, experiments with compounds which induce the Ya GST subunit (phenobarbital, 3-methylcholanthrene, and ß-naphtoflavone) in mouse and rat hepatoma cells did not cause an upregulation of Ya transcription in the human Hep G2 line (115). To elucidate if mutagenicity assays with Hep G2 cells reflect the DNA-protective effects of GST inducers, we carried out preliminary experiments with diallyldisulfide (DADS), which is a constituent of *Allium* vegetables. Allylsulfides are potent inducers of GST in rodents and block tumor formation by B(a)P in mice (Sparnins *et al.*, 1988; Wattenberg *et al.*, 1989; Gudi and Singh, 1991). The results shown in Figure 3 indicate that pretreatment of Hep G2 cells with 5µM DADS leads to a 1.5- fold enhancement of the GST activity, that the induction of MN by B(a)P in DADS preexposed cells is abolished and the tail lenghts of B(a)P induced comets are reduced (Habig *et al.*, 1974).

Musk ketone (MK) and other musk derivatives are frequently used for cosmetical preparations (Emig *et al.*, 1996). MK causes a moderate (statistically

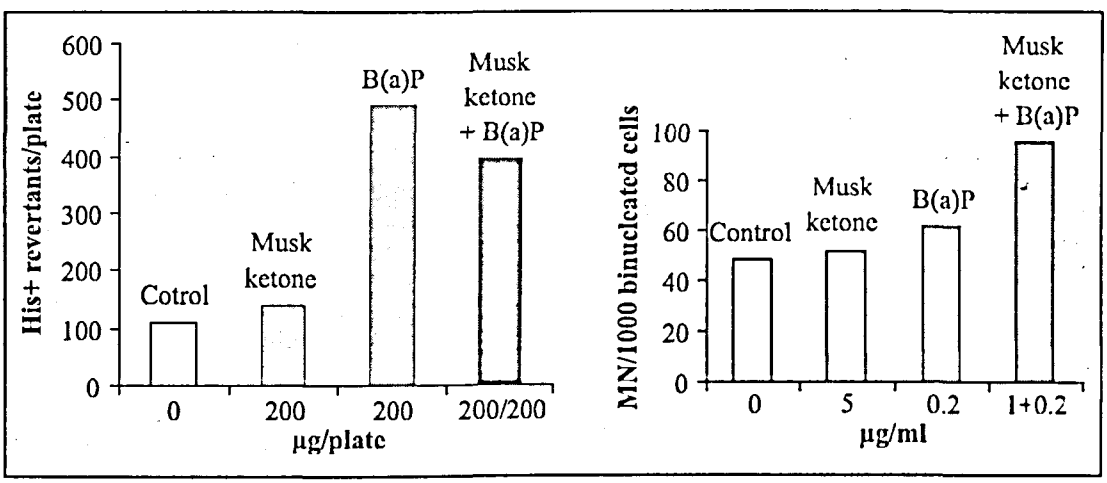

FIGURE 4: *Effect of musk ketone (MK) on the formation of HIS⁺ revertants in* Salmonella *strain TA100 and on the formation of MN in Hep G2 cells. Bacterial assays were carried out as plate incorporation assays with rat liver S9 mix [75], MK was plated directly with B(a)P and the indicator cells. MN assays were carried out as described (Natarajan and Darroudi, 1981). Before addition of cytochalasin B, the cells were preincubated for 48 hrs with MK, washed and exposed for another 28 hrs to B(a)P. In experiments with bacterial indicators 3 plates were evaluated per experimental point, in MN tests 2000 binucleated cells were enumerated per point.*

not significant) decrease of B(a)P induced HIS$^+$ revertants in *Salmonella*/microsome tests (Fig. 4), but recent findings indicated that S9 liver homogenates of rats, which have been pretreated with MK, have an increased activation capacity for PAHs (including B(a)P). This effect is apparently due to induction of cytochrome P-450 IA1 (Mersch-Sundermann, 1996). Our experiments show that the frequency of B(a)P induced MN in Hep G2 cells is almost doubled upon pretreatment of the cells with 1 mM MK. This indicates that the MN/Hep G2 assays reflect the *in vivo* situation and that incorrect results obtained in conventional *in vitro* assays with exogenous metabolic activation mixtures can be avoided.

Table 5 contains further data from other antimutagenicity studies. Salvadori *et al.*, (1993) investigated the protective effects of b-carotene and found a reduction of CP induced MN formation whereas no such effect was seen in combination with MMC which is a direct acting mutagen.

An interesting report came from Grdina *et al.*, (1992) who found that AZT, an anti-AIDS drug causes an induction of 6TG^r mutants in Hep G2 cells (but not in CHO cells) and that this effect is antagonized by post-treatment with aminothiol. The authors concluded that the antimutagenic effect is due to interaction with DNA-repair processes and suggested that aminothiols might prevent the genotoxic and carcinogenic effects of AZT and structurally related reverse transcriptase inhibitors in humans.

Conclusions

The human hepatoblastoma line Hep G2 has been used since more than 15 years in mutagenicity studies. In contrast to other cell lines used in genetic toxicology, these cells have retained the activities of a number of phase I and phase II enzymes which play an essential role in the activation/detoxification of promutagens/procarcinogens. A number of biochemical studies indicated that some of the drug metabolizing enzymes (various cytochrome P-450 isoenzymes, GST) are inducible. An evaluation of the currently available data bases shows that mutagenicity experiments with Hep G2 cells reflect the effects of genotoxic carcinogens in the living animal better than conventional *in vitro* test procedures with exogenous activation mixtures: (i) Representatives of important classes of carcinogens such as HAAs, PAHs, aflatoxins, aromatic amines and nitrosamines give positive results in tests with Hep G2 cells. (ii) Hep G2 cells enable the detection of genotoxic effects of rodent carcinogens such as safrol and HEMPA which give negative results in experiments with metabolically incompetent indicator cells. (iii) Compounds which are structurally related to carcinogens but are devoid of carcinogenic activity in rodents (pyrene, 4-AAF) give negative results in experiments with Hep G2 cells. (iv) The ranking order of genotoxic potencies of different compound within a class of environmental carcinogens (e.g. substituted PAHs and HAAs) correlates well with their carcinogenic activities in rodent bioassays.

Hep G2 cells have also been used successfully in a number of antimutagenicity studies. Certain mechanisms which can not be detected in tests with metabolically incompetent cells can be identified properly in experiments with the Hep G2 cells. These mechanisms include induction of detoxifyin enzymes, inactivation of DNA-reactive metabolites, and inhibition of activating enzymes. In conclusion, genotoxicity assays with Hep G2 cells appear to be a promising approach for the detection of antimutagens and in the last years fast and cost effective methods (e.g. MN assays, SCGE tests) have been developed which could be used in large scale screening trials.

Important steps for the future acceptance of this assay system will be the harmonization of the cultivation conditions (which play an essential role in the expression of enzyme activities) and standardization of the potential use of enzyme inducers and of the treatment schedules.

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Abbreviations

2-AA, 2-aminoanthracene; 2-AAF, 2-acetylaminofluorene; 4-AAF, 4-acetylaminofluorene; 2-AF, 2-amino-fluorene; 4-AB, 4-aminobiphenyl; AFB_1, aflatoxin B_1; AHH, arylhydrocarbon-hydroxylase; AZT, 3'-acido-3'-deoxythymidine; BA, benzanthracene; B(a)P, benzo(a)pyrene; CHO, Chinese hamster ovary cells; CP, cyclo-phosphamide; DADS, diallyldisulfide; DMBA, 7,12-dimethylbenz(a)anthracene; 1,3-DNP, 1,3-dinitropyrene; 1,6-DNP, 1,6-dinitropyrene; 1,8-DNP, 1,8-dinitropyrene; FMO, flavine containing monooxygenase; GST, glutathione-S-transferase; HAAs, heterocyclic aromatic amines; HMPA, hexamethylphosphoramide; IQ, 2-amino-3-methyl-

3H-imidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MK, musk ketone; MMC, mitomycin C; MMS, methyl-methan-sulfonate; MN, micronucleus; MNNG, methyl-nitro-nitroso-guanidine; NAT, N-acetyltransferase, NBU, N-nitrosobutylurea; NDBA, N-nitrosodibutylamine; NDEA, N-nitrosodiethylamine; NDMA, N-nitrosodimethyl-amine; NDPA, N-nitrosodipropylamine; NEU, ethylnitrosurea; NMOR, N-nitrosomorpholine; 1-NP, 1-nitropyrene; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine; 4-NQO, 4-nitroquinoline-n-oxide; PAHs, polycyclic aromatic hydrocarbons; PhIP, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine; SCE, sister chromatide exchange; SCGE, single cell gel electrophoresis; 6-TG^r, 6-thioguanine resistance; Trp-P-1, 2-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; V-79, Chinese hamster fibroblasts