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SFTG international collaborative study on in vitro micronucleus test I. General conditions and overall conclusions of the study

Elisabeth Lorge [a,*], Veronique Thybaud [b], Marilyn J. Aardema [c], Jo Oliver [d], Akihiro Wakata [e], Giocondo Lorenzon [f], Daniel Marzin [g]

[a] *Servier Group, Drug Safety Assessment, F-45403 Orleans-Gidy, France*
[b] *Sanofi Aventis, Drug Safety Evaluation, F-94400 Vitry-sur-Seine, France*
[c] *The Procter & Gamble Co., Cincinnati, OH 45252, USA*
[d] *GlaxoSmithKline Research & Development, United Kingdom*
[e] *Yamanouchi Pharmaceutical Co., Ltd., Safety Research Laboratories, Tokyo, Japan*
[f] *Prostrakan Pharmaceuticals, F-93230 Romainville, France*
[g] *Institut Pasteur, Laboratoire de Toxicologie, F-59019 Lille, France*

Abstract

This study, coordinated by the SFTG (French branch of European Environmental Mutagen Society), included 38 participants from Europe, Japan and America. Clastogens (bleomycin, urethane), including base and nucleoside analogs (5-fluorouracil and cytosine arabinoside), aneugens and/or polyploidy inducers (colchicine, diethylstilboestrol, griseofulvin and thiabendazole), as well as non-genotoxic compounds (mannitol and clofibrate), were tested. Four cell types were used, i.e. human lymphocytes in the presence of cytochalasin B and CHO, CHL and L5178Y cell lines, in the presence or absence of cytochalasin B, with various treatment-recovery schedules. Mitomycin C was used as a positive control for all cell types.

Mannitol and clofibrate were consistently negative in all cell types and with all treatment-recovery conditions. Urethane, known to induce questionable clastogenicity, was not found as positive. Bleomycin and mitomycin C were found positive in all treatment-recovery conditions. The base and nucleoside analogs were less easy to detect, especially 5-fluorouracil due to the interference with cytotoxicity, while cytosine arabinoside was detected in all cell types depending on the treatment-recovery schedule. Aneugens (colchicine, diethylstilboestrol and griseofulvin) were all detected in all cell types. In this study, the optimal detection was ensured when a short treatment followed by a long recovery was associated with a long continuous treatment without recovery. There was no impact of the presence or absence of cytochalasin B on the detection of micronucleated cells on cell lines. Scoring micronucleated cells in both mononucleated and binucleated cells when using cytochalasin B was confirmed to be useful for the detection and the identification of aneugens.

In conclusion, these results, together with previously published validation studies, provide a useful contribution to the optimisation of a study protocol for the detection of both clastogens and aneugens in the in vitro micronucleus test.

Keywords: In vitro micronucleus assay; Human lymphocytes; CHO cells; CHL cells; L5178Y cells; Cytochalasin B; Clastogen; Aneugen

1. Introduction

The detection of micronuclei has been widely used for years for the evaluation of in vivo mutagenic, clastogenic and aneugenic effects. In the past, the high number

* Corresponding author.
E-mail address: elisabeth.lorge@fr.netgrs.com (E. Lorge).

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of analysable cells, the simplicity of the technique, the possibility of automation, as well as the ability to more accurately detect aneugens, led to prefer the in vivo micronucleus test to the in vivo chromosome aberration test [1–5]. The same advantages are offered by the in vitro micronucleus test, which can also be automated and even miniaturised [6–13]. Promising comparisons of the results obtained in the in vitro micronucleus test and in the in vitro chromosome aberration test are available in the literature and suggest that the in vitro micronucleus test could be used as an alternative to the in vitro chromosome aberration test [14–19].

In vitro micronucleus test was developed at first as a method for the measurement of structural chromosomal damage [20]. Acentric fragments of chromosomes were known to result from chromosomal rearrangements and damage, i.e. clastogenic effects produced by initial mutagenic events. Until recently, only mutagenic and clastogenic endpoints were considered in genotoxicity evaluation. No standard in vitro genotoxicity test was adequate to detect aneugens, susceptible to induce numerical chromosome aberrations. However, numerical chromosome aberrations have been shown to be related to the induction of tumors and process of cell transformation by indirect mechanisms such as genomic instability [21–25] and thus, represent an endpoint to be assessed for a full evaluation of genotoxic potential of a new compound. The micronucleus test can detect clastogens like the chromosome aberration test. However, it is also able to detect chromosome loss [3,26–37], whereas the chromosome aberration test can only provide an indication on polyploidy [38,39]. Additionally, the in vitro micronucleus test allows not only the detection of aneugens as genotoxicants, but also their discrimination from clastogens, by means of complementary techniques such as fluorescent in situ hybridisation (FISH) with centromeric DNA sequences or kinetochore specific staining [40–54]. This is a precious tool in research to better understand the mechanisms of aneuploidy and determine thresholds for aneuploidy [26,29,45,55–59]. More generally, aneuploidy is the best known and most documented example of indirect genotoxicity [60]. The possibility of measuring thresholds may be useful for risk assessment in routine genotoxicity evaluation. Thus, the list of compounds studied here included both clastogens and aneugens. As the in vitro chromosome aberration test and the mouse lymphoma assay are the most currently used models to assess DNA and chromosomal damage for regulatory purposes, compounds where data were published on the in vitro chromosome aberration test and on the mouse lymphoma assay were chosen for the present study.

The in vitro micronucleus test was at first mainly developed and standardised on primary cultures of human lymphocytes [3,20,61–63], especially for biomonitoring studies which broadly involve cytogenetic techniques [64–73]. However, the use of human lymphocytes, especially for screening tests, may be limited by the availability of the cells and by the variability between human donors [74]. Therefore, a wide variety of cells are also used to detect the induction of micronuclei in various fields of investigation, as different as environment or tissue-specific carcinogenesis [75–79]. The suitable cell types for the in vitro micronucleus test also include cell lines [15,80], which are chosen for their growth capabilities. The most usually employed cell lines for screening in industry, are those used in the in vitro chromosome aberration test and are karyotypically stable, namely Chinese Hamster Ovary (CHO) cells [81–85], Chinese Hamster Lung (CHL) cells [32,86–88], V79 hamster cells [8,15,89–91], and, more recently, L5178Y mouse lymphoma cells [92–97]. These were the cell types we compared, with the human lymphocytes, in the present study (see subsequent cell type-specific publications in this issue [98–101]). The cytokinesis-block method has been demonstrated to be the best technique in primary cultures to identify cells which had undergone one cell division [102–106]. As human lymphocytes require a mitogenic stimulation to divide in culture, it is needed to make sure analysed cells have completed a division [20]. However, the use of cytochalasin B for actively growing cells, such as CHO, CHL, V79 and L5178Y cells, is questionable. Moreover, the occurrence of cytochalasin B-induced artefacts has been reported [107] and it was also pointed out that its concentration is critical in this test [108], adding a source of variability in the system. As the need for cytochalasin B is still controversial for actively dividing cell lines, it was one of the conditions evaluated in this collaborative study.

In recent years, the duration of exposure in the in vitro genotoxicity testing has been shown to be critical in detecting every kind of genotoxic compound. It has been demonstrated that certain chemicals, such as spindle poisons or nucleotide analogs are more easily detected by longer treatments (covering a complete cell cycle) or delayed sampling times in the chromosome aberration test [109], and in the mouse lymphoma assay [110–113]. Therefore, one of our objectives in this study was to determine which combinations of treatment and recovery times have to be used to detect every kind of genotoxic compounds.

The in vitro micronucleus test is today considered as a usual tool for genotoxicity assessment all over

the world, especially in the industry, for screening purposes. Recently, numerous validation studies, comparative evaluations of available data or surveys of test practice have been published [14,16,17,86–90,114–125]. The outcome of the present study, together with these previous papers will contribute to better define a standard protocol for the use of the in vitro micronucleus test.

2. Materials and methods

2.1. General conditions of the collaborative study

The study was coordinated by an organizing committee supported by the SFTG (the French branch of the European Environmental Mutagen Society) and included 38 laboratories from Europe, America and Japan. The participating laboratories are listed in Table 1.

The aim of this collaborative work was to assess the performances of the in vitro micronucleus test under real testing conditions followed in any experienced laboratory for an unknown compound. Thus, each laboratory was left free to use its own technique for slide preparations and to select concentrations to be tested following a preliminary cytotoxicity assay. However, a survey on the most usually used procedures was conducted among the participating laboratories by the organizing committee before the study and the collated informations were used to establish a common protocol for the treatment and recovery schedules. In most cases, each compound was tested independently by two laboratories and when discordant results were obtained, a third laboratory was included whenever possible. Each laboratory, for a given compound, was named Lab 1, Lab 2 or Lab 3. Depending on the number of laboratories able to handle the in vitro micronucleus assay on each cell type, it was unfortunately not possible to test all the compounds on all the cell types. The tested compounds came from the same source and the same batches for all the laboratories and they were coded. The preferred solvents were indicated. The positive control agent was common to all laboratories and was from the same supplier for all the laboratories. The cell lines were obtained from common sources. The treatment and recovery schedules and the conditions for the selection of concentrations to be evaluated were described in the protocol, as well as the conditions for scoring and the parameters to be evaluated.

This study was aimed to evaluate different treatment-recovery schedules and conditions, namely in the presence or absence of cytochalasin B. Therefore, no experiment was conducted with a metabolic activation system, in order to minimize the sources of variability. Moreover, the use of a metabolic activation system was not expected to bring additional information on suitable treatment-recovery conditions. No strict quantitative comparisons were made, as the compounds were tested blindly and therefore, there was no determination of the absolute lowest effective concentration. The comparisons were based on the capacity of each

Table 1
List of participants

Name of the laboratory	Country	Cell type(s) used
ABBOTT LABORATORIES	USA	CHO cells
ASTRA	UK	Human lymphocytes
AVENTIS Pharma	France	L5178Y cells
BIORELIANCE	USA	CHO cells
COVANCE LABORATORIES, Inc.	USA	CHO cells
DAIICHI PHARMACEUTICAL Co., Ltd	Japan	CHL cells
DuPont Pharmaceuticals Company	USA	CHO cells
GLAXO K.K.	Japan	CHO cells
GLAXO WELLCOME R&D	UK	L5178Y cells
HATANO RESEARCH INSTITUTE	Japan	CHL cells
HOECHST MARION ROUSSEL	France	Human lymphocytes
HUNTINGDON LIFE SCIENCES	UK	Human lymphocytes
INSTITUT PASTEUR	France	Human lymphocytes
KAKEN PHARMACEUTICAL Co., Ltd	Japan	CHL cells
KIRIN BREWERY Co., Ltd.	Japan	CHL cells
KISSEI PHARMACEUTICAL Co., Ltd.	Japan	CHL cells
L'OREAL	France	L5178Y cells
MEIJI SEIKA KAISHA Ltd.	Japan	CHL cells
NATIONAL INSTITUTE OF HEALTH SCIENCES	Japan	CHL cells
NOTOX	The Netherlands	Human lymphocytes
NOVARTIS	Switzerland	L5178Y cells
OTSUKA PHARMACEUTICAL Co., Ltd.	Japan	L5178Y cells
PFIZER Inc.	USA	CHO cells
PROCTER &GAMBLE Co.	USA	CHO cells
Research Toxicology Center	Italy	Human lymphocytes
SANOFI-SYNTHELABO	France	L5178Y cells
SEARLE	USA	CHO cells
SERVIER GROUP	France	Human lymphocytes
SHIONOGI & Co., Ltd.	Japan	CHL cells
SS Pharmaceutical Co., Ltd.	Japan	CHL cells
TNO Nutrition and Food Research Institute	The Netherlands	Human lymphocytes
TORAY INDUSTRIES, Inc.	Japan	CHL cells

Table 1 (*Continued*)

Name of the laboratory	Country	Cell type(s) used
TOYAMA CHEMICAL Co., Ltd.	Japan	CHL cells
Universidad Nacional Autonoma de Mexico	Mexico	Human lymphocytes
Universite d'Aix Marseille II	France	Human lymphocytes
YAMANOUCHI PHARMACEUTICAL Co., Ltd.	Japan	CHL cells
YOSHITOMI PHARMACEUTICAL INDUSTRIES, Ltd.	Japan	CHL cells
ZERIA PHARMACEUTICAL Co., Ltd.	Japan	CHL cells

treatment-recovery condition to detect the compound as positive or negative, according to the criteria specified in the protocol.

2.2. Cells

The cells were obtained from common sources and were grown in the appropriate media, described in the subsequent cell type-specific publications in this issue [98–101]. They were maintained according to accepted good scientific practices. Cells were seeded at densities that ensured cells were actively growing at the time of treatment.

2.3. Choice of tested chemicals

The tested compounds were chosen to be well-known compounds, representative of non-genotoxic compounds, clastogens (including base and nucleoside analogs), aneugens and polyploidy inducers. Compounds with available data on other systems were preferred (Tables 2 and 3).

All the compounds were purchased from Sigma–Aldrich Chimie S.A.R.L. (Saint Quentin Fallavier, France), except the mitomycin C used in USA, provided by Sigma–Aldrich Research (Saint-Louis, Missouri, USA) and the mitomycin C used in Japan, provided by Sigma–Aldrich Japan K.K. (Tokyo, Japan). The tested compounds were coded and dispatched to the participants by the organising committee of the study, so that they were tested blindly. The participants were asked to handle each compound with the same care as for a known carcinogen.

2.3.1. Non-genotoxic compounds

2.3.1.1. Mannitol. Mannitol, a typical non-genotoxic non-carcinogen, is clearly negative in all genotoxicity tests where data are available [126,127]. Mannitol was chosen as a representative non-genotoxic non-carcinogen expected to induce no cytotoxicity at high concentration levels.

Table 2
Published data on the selected compounds

Compound	Carcinogenicity	Bacterial reverse mutation	In vitro chromosome aberration	In vitro mammalian cell gene mutation	In vitro micronucleus test	In vivo micronucleus test
D-mannitol	− [126], − [127]	− [126]	− [126]	No data	No data	− [127]
Clofibrate	+ [128]	− [126], − [129]	+ [126]	− [129]	− [78]	No data
Urethane	+ [126], + [146]	− [126]	+ [148], − [149], − [150]	− [110][a], − [111][a], − [112][b,a]	− [89], − [86]	+ [126], + [5], + [147]
Bleomycin	I [127]	− [132], + [133]	+ [126]	+ [130]	+ [89], + [131]	− [134], + [5]
Mitomycin C	+ [126], + [137]	+ [136]	+ [126]	+ [110], + [135]	+ [89], + [86]	+ [126], + [5]
5-Fluorouracil	I [127], − [143], − [144], + [145]	+ [126]	+ [126], + [142]	+ [110], + [111]	Eq [89], + [86]	+ [126], + [5], + [134], + [142]
Cytosine arabinoside	− [140]	+ [138]	+ [126]	+ [110], + [111], + [139]	+ [89]	+ [5], + [134]
Colchicine	− [152]	− [152]	+ [14], + [81]	− [111], + [112][b]	+ [86], + [14]	+ [5], + [152]
Diethylstilboestrol	+ [126], + [153]	− [126], − [156]	+ [126][c], − [14]	+ [110][d], + [111][d], + [155][d], + [154][e]	+ [89], + [86], + [14]	− [126]
Griseofulvin	+ [126]	− [126]	+ [126]	+ [110], [111]	+ [89], + [91]	− [126], I [127]
Thiabendazole	− [158]	− [126], − [164]	− [126], − [14]	− [111], + [112][b]	I [14], + [89]	+ [5]

+: positive; −: negative; I: inconclusive.
[a] Some marginal effects observed.
[b] 24 h treatment in the mouse lymphoma tk assay.
[c] Only numerical aberrations.
[d] Positive only in the presence of S9.
[e] Positive also without S9.

Table 3
List of selected compounds

Compound	CAS No.	Solvent
Non-genotoxic compounds		
D-mannitol	69-65-8	Aqueous solvent
Clofibrate	637-07-0	DMSO
Clastogens		
Bleomycin	9041-93-4	Aqueous solvent
Mitomycin C[a]	50-07-7	Aqueous solvent
5-Fluorouracil	51-21-8	Aqueous solvent
Cytosine arabinoside	147-94-4	Aqueous solvent
In vitro equivocal compound		
Urethane	51-79-6	Aqueous solvent
Aneugens		
Colchicine	64-86-8	Aqueous solvent
Diethylstilboestrol	56-53-1	Ethanol
Griseofulvin	126-07-8	DMSO
Thiabendazole	148-79-8	Aqueous solvent

[a] Used as the positive control.

2.3.1.2. Clofibrate. Clofibrate, a well-known non-genotoxic hepatocarcinogen (through a mechanism involving peroxisome proliferation) [128], was shown negative in the main in vitro genotoxicity test systems, including an in vitro micronucleus assay in primary culture of hepatocytes [81,129]. Equivocal results were found in the in vitro chromosomal aberration test on CHL cells [126]. No data were available on the in vivo micronucleus test. Clofibrate was chosen as a classical non-genotoxic carcinogen expected to induce some cytotoxicity.

2.3.2. Clastogens

We chose direct-acting clastogens as well as base and nucleoside analogs known to induce chromosome damage via DNA synthesis inhibition. Moreover, cytosine arabinoside and 5-fluorouracil were selected to evaluate the impact of high cytotoxicity on genotoxicity detection, as interference between both parameters is known to result from the inhibition of DNA synthesis for such compounds.

2.3.2.1. Bleomycin. Bleomycin, a radiomimetic compound, whose activity is S-phase independent, was found positive in the in vitro chromosome aberration test on various cells, in the in vitro mammalian cell gene mutation test and in the in vitro micronucleus test [89,126,130,131]. It also induced gene mutations in the Ames test when oxidant-sensitive strains were used [132,133]. In vivo micronucleus tests in bone marrow gave contradictory results. This lack of clear positive results was attributed to its specificity toward epithelial cells [5,134]. No adequate studies were available for carcinogenicity assessment [127]. Bleomycin represented the S-phase independent clastogen in this study.

2.3.2.2. Mitomycin C. Mitomycin C, a cross-linking agent, demonstrated genotoxicity in all in vitro and in vivo test systems in mammalian cells and animals, including the in vitro micronucleus test [5,86,89,110,126,135]. It was also positive in the Ames test [136]. It was clearly demonstrated as carcinogenic [126,137]. Mitomycin C, chosen for its broad range of genotoxicity effects, was also used as the positive control in all assays of this study.

2.3.2.3. Cytosine arabinoside. Cytosine arabinoside, a nucleoside analog, is another kind of well-known genotoxic compound, positive in various in vitro and in vivo genotoxicity test systems [5,89,110,111,126,134,138,139]. However, cytosine arabinoside was not demonstrated carcinogenic [140]. It acts at very low concentrations. It is known to inhibit the excision repair step [141] and it is also susceptible to interfere with DNA synthesis.

2.3.2.4. 5-Fluorouracil. 5-Fluorouracil also interferes with nucleic acid synthesis. It was clearly genotoxic in all the test systems used [5,86,89,110,111,126,134,142]. Discrepant and inconclusive results were obtained in carcinogenicity studies [127,143–145].

2.3.2.5. In vitro equivocal compound. Urethane was positive in numerous carcinogenicity studies [126,146] and in the in vivo genotoxicity studies [5,126,147], but it was negative in gene mutation assays, including the mouse lymphoma assay even with a 24-h treatment and even with S9 [86,89,110–112,126]. Inconsistent results were obtained in the in vitro chromosomal aberration test [148–150]. The marginal or negative results reported in the in vitro genotoxicity studies might be due to the inability of standard S9 preparations to hydroxylate urethane into its active derivative, the vinyl epoxide intermediate [151]. In this study, urethane was chosen as an in vivo specific genotoxic compound demonstrating equivocal results in other in vitro test systems, in order to evaluate if the results obtained in the present study were consistent with these equivocal results or if any of the conditions tested in this study would clarify the results obtained with urethane.

2.3.3. Aneugens

We included aneugens, most being spindle inhibitors known to induce chromosome loss and/or polyploidy.

2.3.3.1. Colchicine. Colchicine has the typical profile of a non-carcinogenic aneugen. It is negative in gene mutation assays [111,152], except for the mouse lymphoma assay when a 24-h treatment was used [112], and positive in the in vivo and in vitro micronucleus tests [5,14,86,152]. It is known to induce numerical aberrations resulting from the metaphase-blockage by the inhibition of tubulin polymerisation [14,81]. Colchicine was chosen to assess the ability of the in vitro micronucleus test to specifically detect aneugens.

2.3.3.2. Diethylstilboestrol. Diethylstilboestrol, a well-known carcinogen [126,153], was also characterized by a genotoxicity profile of aneugen evidenced by positive

results in the in vitro mouse lymphoma assay (mainly with S9), in the in vitro chromosome aberration test (only numerical aberrations) and in the in vitro micronucleus test [14,86,89,110,111,126,154,155]. It was negative in the bacterial reverse mutation assay and in the in vivo micronucleus test [126,156]. Diethylstilboestrol was shown to act on the polymerisation and depolymerisation of microtubules [157]. Thus, we chose it additionally to colchicine as a model compound to assess the ability of the in vitro micronucleus test to detect various types of aneugens.

2.3.3.3. Griseofulvin. Griseofulvin is also a carcinogen [126] and is positive in all genotoxicity tests except the bacterial reverse mutation assay [89,110,111,126]. It was found inconclusive in the in vivo micronucleus test [126,127]. In the in vitro chromosome aberration test it induced not only numerical aberrations but also structural aberrations. It is thought that the inhibition of microtubule assembly caused by griseofulvin may also result in mechanical breaks, therefore, in structural chromosomal aberrations.

2.3.3.4. Thiabendazole. Thiabendazole also inhibits tubulin assembly. It is a non-carcinogen [158], positive in the in vivo micronucleus test [5], and it was found positive in the mouse lymphoma assay in one collaborative study only with the 24-h treatment [112]. The results obtained in the in vitro chromosome aberration tests and the in vitro micronucleus tests were controversial [14,89,126]. Thiabendazole was unfortunately tested only on human lymphocytes due to the lack of availability of laboratories handling other cell types at the end of the study.

2.4. Solvents and choice of concentrations

The tested compounds, solvents used and CAS numbers are listed in Table 3.

The solvents to use and the conditions of storage were specified. When a solvent other than medium was used (e.g. DMSO), the final concentration of solvent in the culture medium did not exceed 1% (v/v).

In the case of cytotoxic compounds, the highest analysed concentration should produce 50–60% cytotoxicity, the lower limit of 50% being based on guidelines for cytogenetic studies and the upper limit of 60% being supposed to avoid irrelevant results. In the case of non-cytotoxic compounds, the highest concentration should induce a precipitate obvious to the naked eye at the end of the treatment, or was equal to 5000 μg/ml. The concentration levels were separated by no more than a $\sqrt{10}$ factor [159–161].

2.5. Positive control

All the participants used mitomycin C as a positive control. It was purchased from Sigma Chemicals and delivered by the organising committee of the study or by local subsidiaries. It was prepared as an aqueous solution and added to not exceed 1% (v/v) in the culture medium. The concentrations to be used in the main study were based on the results of a preliminary study for each treatment-recovery schedule (see Section 3.2).

2.6. Study design

Two assays were generally performed. The dose range-finding assay, using generally one culture per concentration was considered as the first analysable assay if it also included scoring of micronucleated cells as well as both negative and positive controls. The main assay included two cultures per concentration.

2.7. Culture conditions and slide preparations

Culture conditions and slide preparations for each cell type are described in the specific papers in this issue [98–101].

2.8. Schedules for treatment and recovery before harvest

All the schedules included short or long treatments, and the cells were harvested after a short, long or after no recovery periods after the end of treatment. When cell lines were used, the assays were conducted both with and without cytochalasin B (Table 4). These schedules are described in detail in the specific papers in this issue [98–101].

2.9. Cytotoxicity assessment

When cytochalasin B was used, cytotoxicity was assessed by measuring the incidence of cells with two or more nuclei among the total number of cells. Mononucleated, binucleated and multinucleated cells were recorded separately. A minimum of 1000 cells per culture was scored. Cytotoxicity in treated cultures was assessed as the reduction in the incidence of multinucleated cells among total cells as compared to the concurrent solvent control. This method of cytotoxicity evaluation was the most usual one among the participants of the study at that time, as highlighted by the results of the survey made before the study. Total cell counts were also reported for additional information.

As a simplification, the level of cytotoxicity was expressed as survival, keeping in mind that cytotoxicity results from both cell death and/or growth inhibition, depending on the measurement used for cytotoxicity assessment. Relative survival was calculated as the ratio of the incidence of multinucleated cells in treated cultures versus the incidence of multinucleated cells in the solvent controls, expressed as a percentage.

When cytochalasin B was not used, cytotoxicity was evaluated by counting the cells at the time of harvest, with a haemocytometer or an electronic cell counter. Cytotoxicity in treated cultures was assessed as the reduction in the number of cells as compared to the concurrent solvent control. Relative survival was calculated as the ratio of the number of cells in treated cultures versus the number of cells in the concurrent vehicle controls, expressed as a percentage.

Table 4
Treatment and recovery times

	Without cytochalasin B				With cytochalasin B		
	Short[a]		Long[a]		Short[a]		Long[a]
	Short[b]	Long[b]	No[b]	Long[b]	Long[b]	Short[b]	Long[b]
Human lymphocytes	nt	nt	nt	nt	$3^c + 45^d$	$3^c + 26^d$	$20^c + 28^d$
CHO cells	$3^c + 21^d$	$3^c + 45^d$	$24^c + 0^d$	$24^c + 24^d$	nt	$3^c + 20^d$	$24^c + 20^d$
CHL cells	$3^c + 21^d$	$3^c + 45^d$	$24^c + 0^d$	$24^c + 24^d$	nt	$3^c + 18^d$	$24^c + 18^d$
L5178Y cells	$3^c + 21^d$	$3^c + 45^d$	$24^c + 0^d$	$24^c + 24^d$	nt	$3^c + 20^d$	$24^c + 20^d$

nt: not tested.
[a] Treatment.
[b] Recovery.
[c] Treatment period (h).
[d] Recovery period (h).

Any abnormality (i.e. changes in cell morphology, pycnotic nuclei, apoptotic cells) was noted [30,60,162].

2.10. Genotoxicity assessment

All slides including those of positive and solvent controls were coded before analysis and scored blindly for the evaluation of genotoxicity.

The criteria used for identifying micronuclei fulfilled those recommended by the HUMN work [163]. They were as follows:

- area < 1/3 the main nucleus area,
- no overlapping with the nucleus (distinct borders),
- same aspect as the chromatin.

If nucleus fragmentation was observed (no main nucleus), it was scored separately, as an abnormality.

When cytochalasin B was used, micronuclei were scored in 1000 binucleated cells per culture (2000 cells per concentration). When possible, micronuclei were also scored separately in 1000 mononucleated cells per culture (2000 per concentration), or in the highest number of analysable mononucleated cells on the slide (at least 250 per culture, i.e. 500 per concentration) [29,164].

In the absence of cytochalasin B, micronuclei were scored in 1000 cells per culture (2000 cells per concentration).

2.11. Report and evaluation of results

Results were collated on a standard template. The number of micronucleated cells per 1000 cells was reported for each concentration. When two cultures were prepared, the mean of two values was calculated and tabulated.

The genotoxicity induction factor was calculated as the ratio of the incidence of micronucleated cells in treated cultures compared to the incidence of micronucleated cells in the concurrent solvent controls, expressed as a percentage. The comparison of the numbers of micronucleated cells in treated and solvent control cultures was done using the statistical analysis, i.e. the chi-square test.

The magnitude of the response (maximal factor induction) was also calculated and reported.

To compare the treatment conditions, we calculated the sensitivity as the ratio of the genotoxicity induction factor (IF) to the cell survival reflecting the balance between micronuclei induction and cytotoxicity (based on cell counts or on the percentage of binucleated cells in cytochalasin B-treated cultures). Comparisons were only possible for the schedules evaluated both in the presence and in the absence of cytochalasin B in the same assay and at the same concentrations of the tested compound, namely the short treatment followed by the short recovery period, and the long treatment followed by the long recovery period. The ratio IF/survival was calculated for each concentration, when the compound was tested in common under both conditions. In the cytochalasin B-treated cultures, only micronucleated binucleated cells were considered.

To visualise the sensitivity, relatively to the use of cytochalasin B, the results obtained in the short and long treatments as described above with cytochalasin B were plotted on a graph against the results obtained in the same assay with the corresponding treatment-recovery schedules without cytochalasin B. On these graphs, each dot represents one concentration of a given compound for each treatment time. Different symbols were used to differentiate the assays. If the results obtained in the presence or absence of cytochalasin B correlated well, the dots should follow a bisecting line. This representation was done for CHL and L5178Y cell types, separately for clastogens and aneugens. When useful, the coefficient of correlation was calculated (not reported).

2.12. Criteria for the acceptability of assays

The assay acceptance criteria described in the protocols were:

- a statistically significant increase in the incidence of micronucleated cells in positive controls compared to solvent control,

- at least one concentration inducing between 50 and 60% cytotoxicity or, in case of a non-cytotoxic compound, either observation at the highest concentration of a precipitate obvious to the naked eye at the end of the treatment, or a maximum concentration of 5000 μg/ml,
- at least four concentrations analysable for genotoxicity assessment in at least one assay.

If the first criterion was not achieved (no concurrent positive controls), the assay was rejected from the individual data tables, as not properly conducted (see the specific papers in this issue [98–101]).

If one of the two other criteria was not achieved (no rationale for the selection of the highest concentration, less than four concentrations), the assay was reported in the tables of individual data, as properly conducted, but inconclusive and therefore not used for genotoxicity evaluation. However, in cases where a concentration inducing between 50 and 60% reduction in relative survival could not be achieved, even when the assay was repeated at a narrower range of concentrations, the assay was regarded as acceptable if a statistically significant increase in the incidence of micronucleated cells was seen and if at least two concentrations were available for analysis. When no statistically significant increase in the incidence of micronucleated cells was observed even at concentrations producing more than 60% reduction in relative survival, the assay was considered as acceptable if at least three analysable concentrations were available at cytotoxicity lower than 50%.

2.13. Criteria for the interpretation of results

An independent assay was considered as positive when the two following criteria were met:

- a statistically significant increase in the incidence of micronucleated cells over the solvent control (in mononucleated cells when cytochalasin B was not used and either in binucleated or in mononucleated cells when cytochalasin B was used),
- a concentration-dependent effect (no statistical test was used).

An independent assay was considered as negative when the assay acceptance criteria were fulfilled and no increase in the incidence of micronucleated cells compared to the solvent controls was observed.

If a significant increase in the incidence of micronucleated cells was seen without a concentration-dependent effect or at only one concentration, the assay was concluded as equivocal. In any case, a positive response seen only at concentrations inducing more than 60% cytotoxicity was reported in the specific publications in this issue [98–101] but not taken into account for the evaluation of the compound, unless specified in the tables of individual data.

The final conclusion concerning a compound took into account the different assays conducted. The criteria were the following ones:

- the compound was concluded positive when all the accepted assays were positive. If inconsistent results were obtained between assays when the negative assay included too low concentrations or too wide a range of concentrations, only the positive assay was taken into account.
- the compound was concluded negative when all the accepted assays were negative. If an equivocal result was not reproduced in a second assay with an adequate range of concentrations, the compound was classified as negative.
- the compound was considered equivocal when conflicting results were obtained between assays. If a positive response was not confirmed in a second assay, both assays being conducted with an adequate range of concentrations, the compound was classified as equivocal.

3. Results

3.1. Spontaneous background of micronucleated cells

The values for the solvent controls obtained in the main assays are summarized in Table 5 and Fig. 1 (see individual data in the subsequent specific papers in this issue [98–101]). A wide range of spontaneous levels of micronucleated cells was noted among the laboratories for all the cell types, resulting from both intra- and inter-laboratory variability. A comparison of the spontaneous number of micronucleated cells obtained with the main solvents used in CHL and L5178Y cells is presented in Table 6. These data suggested a higher spontaneous rate in the presence of DMSO as compared to aqueous solvents, especially for the L5178Y cells.

3.2. Positive control (mitomycin C)

The concentrations of mitomycin C used in the main studies were selected based on the results obtained in a preliminary study for each cell type and on a compromise between genotoxicity and cytotoxicity. The chosen concentrations for mitomycin C as a positive control and the data obtained in the main studies were collated and summarised in Table 7 (see also individual data in the subsequent specific papers in this issue [98–101]). In all the cell types, at the concentration selected for each schedule, mitomycin C induced high levels of micronucleated cells and low to moderate cytotoxicity. When cytochalasin B was used for cell lines, the same concentrations as in the corresponding schedules without cytochalasin B were chosen, to allow comparisons.

3.3. Tested compounds

The results obtained on the different cell types are summarised in Tables 8–12 (see details and indi-

Table 5
Number of spontaneous micronucleated cells for the different schedules for treatment and recovery

	Without cytochalasin B[a]				With cytochalasin B[b]		
Human lymphocytes	nt	nt	nt	nt	3[c] + 26[d] **7.4** ± 7.5 [0.0–32.6]	3[c] + 45[d] **7.1** ± 5.3 [0.5–19.0]	20[c] + 28[d] **6.9** ± 4.9 [0.3–18.0]
CHO cells	3[c] + 21[d] **10.2** ± 7.0 [0.5–22.0]	3[c] + 45[d] **12.9** ± 8.7 [1.0–26.5]	24[c] + 0[d] **12.4** ± 6.4 [1.5–25.0]	24[c] + 24[d] **13.2** ± 9.5 [0.0–32.5]	3[c] + 20[d] **12.7** ± 6.9 [0.0–24.0]		24[c] + 20[d] **12.9** ± 6.2 [1.5–24.5]
CHL cells	3[c] + 21[d] **11.0** ± 4.7 [3.5–23.0]	3[c] + 45[d] **11.3** ± 5.7 [4.0–29.0]	24[c] + 0[d] **10.6** ± 4.6 [2.5–24.0]	24[c] + 24[d] **11.1** ± 5.0 [4.5–28.0]	3[c] + 18[d] **14.8** ± 6.8 [6.5–34.0]		24[c] + 18[d] **13.1** ± 5.9 [5.0–26.5]
L5178Y cells	3[c] + 21[d] **4.5** ± 3.2 [0.5–14.0]	3[c] + 45[d] **6.4** ± 7.8 [1.0–31.5]	24[c] + 0[d] **3.8** ± 2.5 [0.5–8.0]	24[c] + 24[d] **3.4** ± 3.8 [1.0–12.5]	3[c] + 20[d] **11.5** ± 7.7 [5.0–34.0]		24[c] + 20[d] **8.2** ± 8.0 [1.5–26.5]

nt: not tested. Mean number of micronucleated cells per 1000 cells ± standard deviation. Values in square brackets represent the range of individual data (defined as the control value of each assay). Number of individual data for each cell type and treatment-recovery schedule: 13–29.
[a] In mononucleated cells.
[b] In binucleated cells.
[c] Treatment period (h).
[d] Recovery period (h).

vidual values in the specific papers in this issue [98–101]).

3.3.1. Non-genotoxic compounds

As expected, both mannitol and clofibrate were found negative in all treatment conditions and cell types used (Table 8). Additionally, mannitol was found non-cytotoxic up to 5000 μg/ml while clofibrate showed cytotoxic effects (see individual values in the specific papers in this issue [98–101]).

3.3.2. Clastogens

Bleomycin and mitomycin C induced clear increases in the incidence of micronucleated cells in the four cell types, irrespective of the treatment condition (Table 9). With bleomycin, the genotoxic effects were associated with low or no cytotoxicity. Moreover, micronuclei were detected both in mono and binucleated cells with bleomycin in all cell types and treatment schedules. These micronucleated mononucleated cells may represent the cells which had completed their division before the exposure to cytochalasin B, as bleomycin is cell-cycle independent.

5-Fluorouracil was found positive in almost all the treatment conditions in CHL cells, although results obtained after long recoveries without cytochalasin B remained equivocal (Table 10). On the other hand, this compound was hardly detected in human lymphocytes. In L5178Y cells, the activity of 5-fluorouracil was difficult to demonstrate, due to the interference of cytotoxicity with genotoxicity. Marked increases in the incidence of micronucleated cells were prefer-

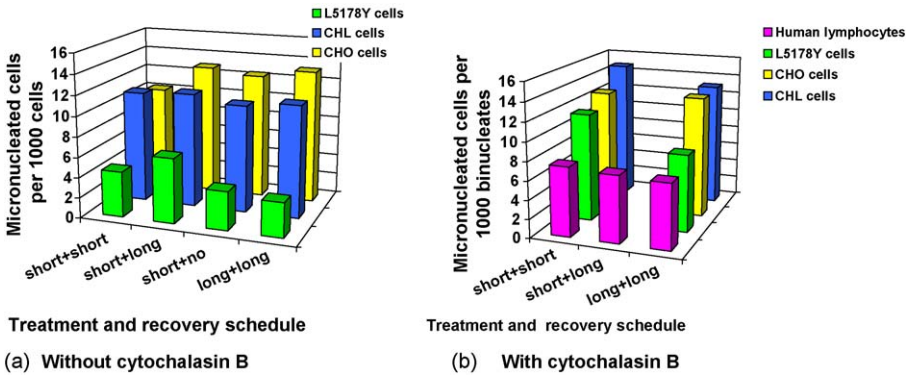

Fig. 1. Levels of spontaneous micronucleated cells: (a) without cytochalasin B and (b) with cytochalasin B.

Table 6
Levels of spontaneous micronucleated cells pending on the solvents used in L5178Y and in CHL cells

		Without cytochalasin B[a]				With cytochalasin B[b]	
L5178Y cells	All solvents	3[c] + 21[d] **4.5** ± 3.2 [0.5–14.0]	3[c] + 45[d] **6.4** ± 7.8 [1.0–31.5]	24[c] + 0[d] **3.8** ± 2.5 [0.5–8.0]	24[c] + 24[d] **3.4** ± 3.8 [1.0–12.5]	3[c] + 20[d] **11.5** ± 7.7 [5.0–34.0]	24[c] + 20[d] **8.2** ± 8.0 [1.5–26.5]
	Aqueous solvents	3[c] + 21[d] **3.6** ± 2.2 [0.5–7.0]	3[c] + 45[d] **4.0** ± 2.4 [1.0–9.0]	24[c] + 0[d] **3.1** ± 2.4 [0.5–8.0]	24[c] + 24[d] **2.5** ± 2.2 [1.0–7.0]	3[c] + 20[d] **8.0** ± 2.6 [5.0–12.5]	24[c] + 20[d] **5.2** ± 2.3 [1.5–9.0]
	DMSO	3[c] + 21[d] **6.0** ± 4.6 [3.0–14.0]	3[c] + 45[d] **13.0** ± 13.5 [2.0–31.5]	24[c] + 0[d] **6.0** ± 2.2 [3.5–7.5]	24[c] + 24[d] **5.1** ± 5.7 [1.0–12.5]	3[c] + 20[d] **17.1** ± 10.1 [6.5–34.0]	24[c] + 20[d] **12.9** ± 11.6 [3.0–26.5]
CHL cells	All solvents	3[c] + 21[d] **11.0** ± 4.7 [3.5–23.0]	3[c] + 45[d] **11.3** ± 5.7 [4.0–29.0]	24[c] + 0[d] **10.6** ± 4.6 [2.5–24.0]	24[c] + 24[d] **11.1** ± 5.0 [4.5–28.0]	3[c] + 20[d] **14.8** ± 6.8 [6.5–34.0]	24[c] + 20[d] **13.1** ± 5.9 [5.0–26.5]
	Aqueous solvents	3[c] + 21[d] **10.6** ± 4.8 [3.5–23.0]	3[c] + 45[d] **10.8** ± 5.9 [4.0–29.0]	24[c] + 0[d] **9.9** ± 4.5 [2.5–19.0]	24[c] + 24[d] **9.4** ± 3.5 [4.5–17.0]	3[c] + 20[d] **14.5** ± 7.6 [6.5–34.0]	24[c] + 20[d] **12.1** ± 6.4 [5.0–26.5]
	DMSO	3[c] + 21[d] **13.2** ± 5.2 [7.5–21.5]	3[c] + 45[d] **13.9** ± 4.1 [11.0–20.0]	24[c] + 0[d] **13.2** ± 5.4 [9.0–24.0]	24[c] + 24[d] **15.6** ± 7.0 [11.0–28.0]	3[c] + 20[d] **14.7** ± 3.3 [11.5–20.0]	24[c] + 20[d] **15.9** ± 5.9 [13.0–26.5]

nt: not tested. Mean number of micronucleated cells per 1000 cells ± standard deviation. Values in square brackets represent the range of individual data (defined as the control value of each assay). Number of data for each cell type and treatment and recovery schedule: 13–29.

[a] In mononucleated cells.

[b] In binucleated cells.

[c] Treatment period (h).

[d] Recovery period (h).

ably noted after long recoveries in L5178Y cells [141].

Cytosine arabinoside was clearly detected as positive in CHO and CHL cells using all the treatment and recovery schedules both in the presence and the absence of cytochalasin B (Table 10). No data is available on L5178Y cell line. In human lymphocytes, cytosine arabinoside was found negative after the 3-h treatment followed by a short recovery period (21 h). On the contrary, it was unequivocally found positive after the 3-h treatment followed by a long recovery period (45 h). Moreover, conflicting results were obtained when the long treatment was applied.

The results obtained with both 5-fluorouracil and cytosine arabinoside pointed out the difficulty of detecting genotoxic compounds at acceptable cytotoxicity levels.

3.3.3. *In vitro equivocal compound*

Urethane was found negative in all treatment conditions and all the cell types used, except in CHL cells where an equivocal result was obtained after long treatment followed by a long recovery period (Table 11). Indeed, there is accumulating evidence that this compound might not be positive in the in vitro genotoxicity tests.

3.3.4. *Aneugens*

Colchicine was unambiguously found positive in CHL cells and human lymphocytes in all the treatment schedules in the presence or absence of cytochalasin B (Table 12). In L5178Y cells, in long-term treatments, the colchicine activity was not easily detected without cytochalasin B, while it was clearly detected with cytochalasin B. Colchicine was not evaluated in CHO cells. In all cases, equivocal results were obtained after short treatments. As expected [29,164], increases in the incidence of micronucleated cells were found in both mono and binucleated cells (Figs. 2 and 3). The former possibly being cells which escaped from mitotic arrest by mitotic slippage. Nevertheless, considering the marked increase in the number of cells in metaphasis, colchicine would not have been missed in mouse lymphoma L5178Y cells, showing that the evaluation of different endpoints is a strength of the micronucleus assay compared to other in vitro test systems.

Diethylstilboestrol clearly increased the incidence of micronucleated human lymphocytes after short treatment followed by a short recovery (Table 12). But the extension of the treatment period or the recovery period resulted in negative or equivocal responses. In CHO and CHL cells, all the treatment schedules revealed genotoxic activity in the presence or absence of cytochalasin

Table 7
Number of micronucleated cells induced by mitomycin C in the main study for the different treatment-recovery schedules

	Without cytochalasin B[a]				With cytochalasin B[b]		
Human lymphocytes	nt	nt	nt	nt	3[c] + 26[d] **54** ± 39 [13–139] *79* ± *18*	3[c] + 45[d] **50** ± 36 [13–142] *81* ± *21*	20[c] + 28[d] **45** ± 27 [6–104] *80* ± *22*
MMC (μg/ml)					0.5	0.25	0.05
CHO cells	3[c] + 21[d] **102** ± 59 [12–188] *66* ± *21*	3[c] + 45[d] **101** ± 67 [10–220] *87* ± *27*	24[c] + 0[d] **118** ± 52 [8–190] *58* ± *17*	24[c] + 24[d] **183** ± 111 [32–451] *60* ± *21*	3[c] + 20[d] **146** ± 93 [49–329] *88* ± *20*		24[c] + 20[d] **382** ± 222 [166–832] *93* ± *21*
MMC (μg/ml)	0.2	0.2	0.1	0.1	0.2		0.1
CHL cells	3[c] + 21[d] **73** ± 24 [38–131] *86* ± *27*	3[c] + 45[d] **48** ± 17 [21–88] *92* ± *26*	24[c] + 0[d] **127** ± 35 [49–193] *79* ± *20*	24[c] + 24[d] **280** ± 94 [168–495] *66* ± *21*	3[c] + 18[d] **93** ± 38 [38–190] *91* ± *9*		24[c] + 18[d] **316** ± 103 [181–500] *105* ± *23*
MMC (μg/ml)	0.1	0.1	0.05	0.05	0.1		0.05
L5178Y cells	3[c] + 21[d] **60** ± 37 [20–133] *88* ± *14*	3[c] + 45[d] **78** ± 57 [29–216] *87* ± *14*	24[c] + 0[d] **70** ± 32 [21–144] *75* ± *18*	24[c] + 24[d] **87** ± 44 [34–150] *82* ± *15*	3[c] + 20[d] **103** ± 34 [42–157] *74* ± *25*		24[c] + 20[d] **131** ± 62 [41–240] *72* ± *25*
MMC (μg/ml)	0.125	0.5	0.0625	0.0625	0.125		0.0625

nt: not tested. MMC: Mitomycin C concentration for the positive control. In bold: mean number of micronucleated cells per 1000 cells ± standard deviation for the corresponding MMC concentration. Values in square brackets represent the range of individual data (defined as the control value of each assay). In italics: mean relative survival (based on cell counts, or on the percentage of binucleated cells for cytochalasin B-treated cultures) ± standard deviation for the corresponding MMC concentration. Number of data for each cell type and treatment and recovery schedule (only accepted assays): 8–29.

[a] In mononucleated cells.
[b] In binucleated cells.
[c] Treatment period (h).
[d] Recovery period (h).

Table 8
Summary of results with non-genotoxic compounds

Compound CAS. No.	Cell type	Without cytochalasin B				With cytochalasin B		
		Short, short[a]	Short, long[a]	Long, no[a]	Long, long[a]	Short, short[a]	Short, long[a]	Long, long[a]
D-mannitol 69-65-8	Human lymphocytes	nt	nt	nt	nt	–	–	–
	CHO cells	–	–	–	–	–	nt	–
	CHL cells	–	–	–	–	–	nt	–
	L5178Y cells	–	–	–	–	–	nt	–
Clofibrate 637-07-0	Human lymphocytes	nt	nt	nt	nt	–	–	–
	CHL cells	–	–	–	–	–	nt	–

nt: not tested; −: compound negative in the treatment and recovery schedule; +: compound positive in the treatment and recovery schedule; +: equivocal response in the treatment and recovery schedule; I: inconclusive (insufficient data to conclude due to the rejection of assays where a genotoxic concentration could not be find at an acceptable cytotoxicity); corresponding individual data are detailed in the specific publications in this issue [98–101].

[a] Treatment, recovery.

Table 9
Summary of results with clastogens

Compound CAS. No.	Cell type	Without cytochalasin B				With cytochalasin B		
		Short, short[a]	Short, long[a]	Long, no[a]	Long, long[a]	Short, short[a]	Short, long[a]	Long, long[a]
Bleomycin 9041-93-4	Human lymphocytes	nt	nt	nt	nt	+	+	+
	CHO cells	+	+	+	+	+	nt	+
	CHL cells	+	+	+	+	+	nt	+
	L5178Y cells	+	+	+	+	+	nt	+
Mitomycin C 50-07-7	Human lymphocytes	nt	nt	nt	nt	+	+	+
	CHO cells	+	+	+	+	+	nt	+
	CHL cells	+	+	+	+	+	nt	+
	L5178Y cells	+	+	+	+	+	nt	+

See Table 8 for legend.
[a] Treatment, recovery.

Table 10
Summary of results with nucleoside and base analogs

Compound CAS. No.	Cell type	Without cytochalasin B				With cytochalasin B		
		Short, short[a]	Short, long[a]	Long, no[a]	Long, long[a]	Short, short[a]	Short, long[a]	Long, long[a]
5-Fluorouracil 51-21-8	Human lymphocytes	nt	nt	nt	nt	I	±	−
	CHL cells	+	±	+	±	+	nt	+
	L5178Y cells	±	+	−	±	−	nt	+
Cytosine arabinoside 147-94-4	Human lymphocytes	nt	nt	nt	nt	−	+	±
	CHO cells	+	+	+	+	+	nt	+
	CHL cells	+	+	+	+	+	nt	+

See Table 8 for legend.
[a] Treatment, recovery.

B though, in CHL cells, some responses remained equivocal after short-term treatments. Diethylstilboestrol was not evaluated in L5178Y cells.

Griseofulvin was demonstrated positive in all experimental conditions in CHL and L5178Y cells, except in L5178Y cells when a short treatment was followed by a long recovery. Moreover, after the short treatment followed by a short recovery, both in the presence or absence of cytochalasin B, the effect in L5178Y was less marked than in CHL cells, resulting in equivocal results. Griseofulvin was not evaluated in human lymphocytes and CHO cells.

Table 11
Summary of results with the in vitro equivocal compound

Compound CAS. No.	Cell type	Without cytochalasin B				With cytochalasin B		
		Short, short[a]	Short, long[a]	Long, no[a]	Long, long[a]	Short, short[a]	Short, long[a]	Long, long[a]
Urethane 51-79-6	Human lymphocytes	nt	nt	nt	nt	−	−	−
	CHO cells	−	−	−	−	−	nt	−
	CHL cells	−	−	−	−	−	nt	±

See Table 8 for legend.
[a] Treatment, recovery.

Table 12
Summary of results with aneugens and polyploidy inducers

Compound CAS. No.	Cell type	Without cytochalasin B				With cytochalasin B		
		Short, short[a]	Short, long[a]	Long, no[a]	Long, long[a]	Short, short[a]	Short, long[a]	Long, long[a]
Colchicine 64-86-8	Human lymphocytes	nt	nt	nt	nt	+	+	+
	CHL cells	+	+	+	+	+	nt	+
	L5178Y cells	±	±	−	−	±	nt	+
Diethylstilboestrol (DES) 56-53-1	Human lymphocytes	nt	nt	nt	nt	+	−	±
	CHO cells	+	+	+	+	+	nt	+
	CHL cells	±	I	+	+	±	nt	+
Griseofulvin 126-07-8	CHL cells	+	+	+	+	+	nt	+
	L5178Y cells	±	−	+	+	±	nt	+
Thiabendazole 126-07-8	Human lymphocytes	nt	nt	nt	nt	−	−	−

See Table 8 for legend.
[a] Treatment, recovery.

Thiabendazole was only evaluated in human lymphocytes and was found negative. Indeed, conflicting results were reported in the in vitro and in vivo genotoxicity studies, which may suggest that the action of thiabendazole on the mitosis may result in acytokinesis cells and therefore would not result in micronuclei formation, but this hypothesis needs more experiments to be verified [3,5,14,26,33,89,165].

4. Discussion

4.1. Tested compounds

The presumed negative compounds did not increase the number of micronucleated cells, irrespective of the cell types exposed, the treatment schedules used, and the presence or absence of cytochalasin B. This result indi-

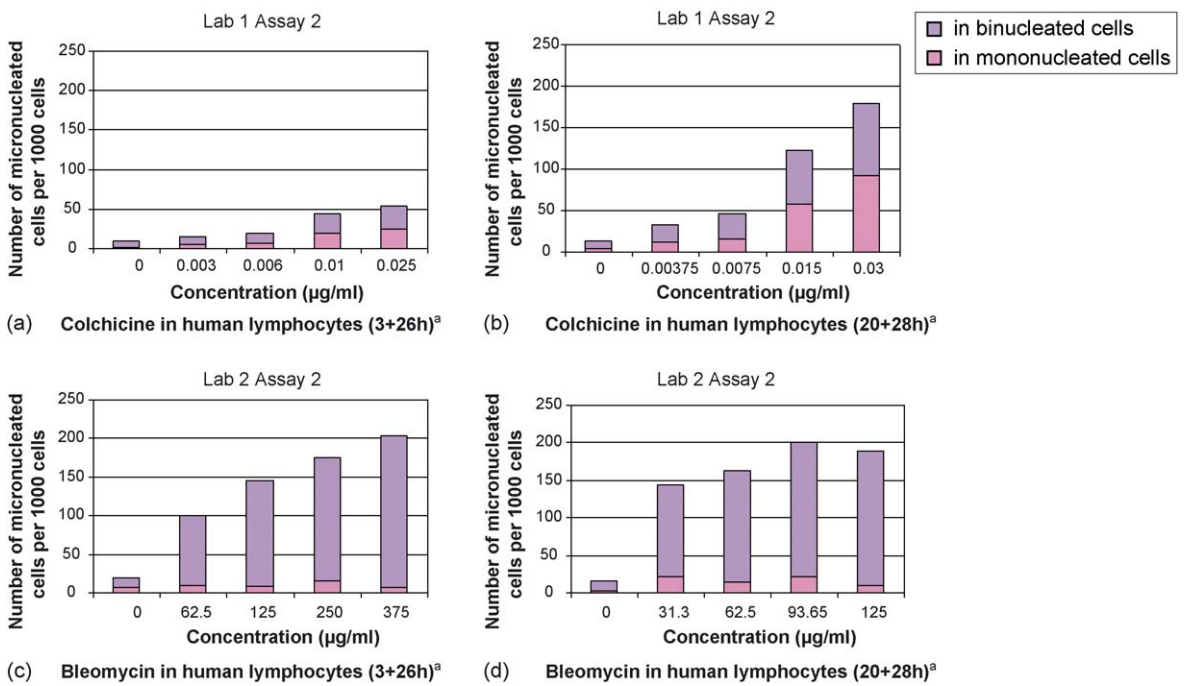

Fig. 2. Micronucleated cell counts in mono and binucleated cells in human lymphocytes: (a) and (b) with colchicine; (c) and (d) with bleomycin. [a] treatment period + recovery period.

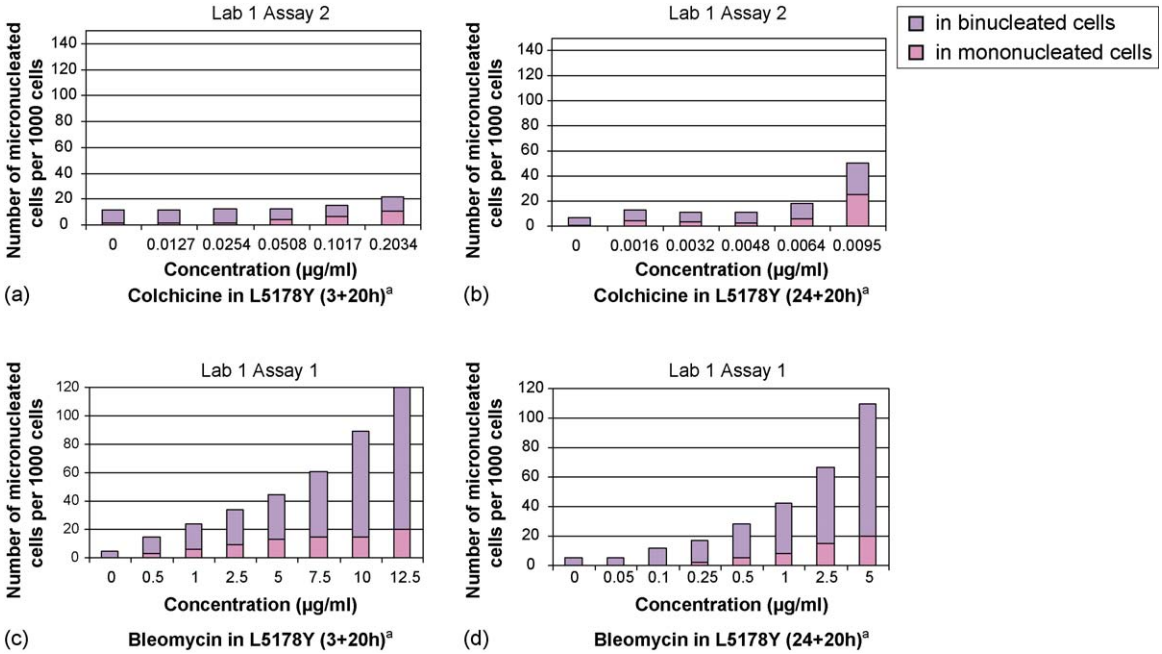

Fig. 3. Micronucleated cell counts in mono and binucleated cells in L5178Y in the presence of cytochalasin B: (a) and (b) with colchicine; (c) and (d) with bleomycin. [a] treatment period + recovery period.

cates a satisfactory specificity of the in vitro micronucleus test. Urethane was classified as an in vitro inconclusive compound on the basis of the literature and it was confirmed as such in the present study. The well-known clastogens, bleomycin and mitomycin C, were unambiguously detected in all cell types and treatment-recovery conditions. Diethylstilboestrol and griseofulvin were not missed in this system with appropriate treatment-harvest schedules. The base and nucleoside analogs, 5-fluorouracil and cytosine arabinoside, as well as the aneugens, colchicine, and thiabendazole (in human lymphocytes), were less easily detected, generally due to the interference of cytotoxicity (cell-cycle slowing down or mitotic arrest) with the evaluation of genotoxicity (for more details, see the specific publications in this issue [98–101]). However, no compound would have been missed, providing the appropriate combination of treatment-recovery schedules, associating a short and a long treatment had been selected. The only exception is thiabendazole on human lymphocytes, whose mechanism needs further clarification.

4.2. Schedules for treatment and recovery

Despite the limited number of compounds evaluated in the present study, the percentages of concordance were calculated to highlight the overall performance of the test in Table 13. The number of concordant results (i.e. presumed negative concluded negative in the study and presumed positive concluded positive in the study) was calculated for each cell type and condition of treatment to determine the most appropriate combination of treatments. Urethane was classified as an in vitro equivocal compound on the basis of the literature and confirmed as such in the present study. Therefore, to avoid introducing a bias in the comparison between the cell types and the schedules urethane was discarded from the evaluation. The results of thiabendazole were also not used for this evaluation, as only obtained in human lymphocytes. In Table 13, concordant (positive and negative) and equivocal results were recorded separately for each cell type and schedule. All presumably positive compounds classified as equivocal were also scored as concordant because this result, in usual toxicology practice, constitutes an alert in genotoxicity assessment and generally leads to confirmatory assays to clarify the results.

No discordant results were found using in CHO and CHL cell lines the combination of short- and long-treatments with any recovery period. Only equivocal responses decreased the percentage of concordance on one occasion in CHL cells, but the limited number of tested compounds precludes to emphasise a significant difference in sensitivity between CHL and CHO cells, with or without cytochalasin B. However, in L5178Y

Table 13
Suitable combinations of treatment-recovery schedules by cell types

	Without cytochalasin B								With cytochalasin B			
	SS + LL		SL + LL		SS + L		SL + L		SS + LL		SS + SL + LL	
Evaluation of the compound	+ and −	Eq	+ and −	Eq	+ and −	Eq	+ and −	Eq	+ and −	Eq	+ and −	Eq
CHO cells	100%	0%	100%	0%	100%	0%	100%	0%	100%	0%		
CHL cells	100%	0%	89%	11%	100%	0%	100%	0%	100%	0%		
L5178Y cells	67%	33%	83%	17%	67%	33%	83%	17%	100%	0%		
Human lymphocytes	nt	nt	nt	nt	nt	nt	nt				75%	13%

SS: short treatment and short recovery; SL: short treatment and long recovery; L: long treatment and no recovery; LL: long treatment and recovery; + and −: clear positive or negative results in at least one of the schedules of the combination; Eq: equivocal results. nt: not tested. Percentage of concordant results with the initial classification, excluding urethane (see Section 4) and thiabendazole (tested only in human lymphocytes); inconclusive results were not taken into account.

cells, equivocal responses were found for all the schedules without cytochalasin B, due to colchicine in each schedule and due to 5-fluorouracil when short treatments were followed by a short recovery. With cytochalasin B, only the long treatment followed by a recovery period allowed the detection of 5-fluorouracil as positive. This suggested that, with or without cytochalasin B, the omission of an extended recovery period after the short or the long treatment would preclude the detection of the compounds with genotoxicity-related cytotoxicity. The association of a short treatment followed by a long recovery and a long treatment allowed to accurately classify all the compounds with CHO, CHL and L5178Y cell lines. Colchicine induced equivocal responses only in the mouse lymphoma L5178Y cell line, mostly at cytotoxic concentrations. No additional treatment-recovery schedule would have improved the result.

In human lymphocytes, the three treatment-harvest schedules were needed to detect almost all the tested compounds. For an unknown compound, the optimal detection would be expected from the combination of a short treatment followed by both a short and a long recovery, and a long treatment followed by recovery.

In conclusion to these comparisons, no discordant results were obtained using these combinations described above, except in human lymphocyte with 5-fluorouracil due to inconclusive results related to cytotoxicity. Due to the small difference between the tested conditions, no ideal condition could be strictly defined for any unknown compound. For all cell types, equivocal results were restricted to two compounds: 5-fluorouracil and colchicine, where cytotoxicity or mitotic arrest interfered with the induction of micronuclei. This points out the importance of parallel scoring of metaphases**.** In the evaluation of an unknown compound, such equivocal responses would have generally led to further experiments, so that no positive compound would have been missed.

Overall, in the present study, the most suitable combinations of treatment-recovery schedules were for the cell lines without cytochalasin B a short treatment followed by a long recovery and a long treatment without recovery. With cytochalasin B, the combination of a short treatment followed by a short recovery and a long treatment followed by a long recovery was found suitable. For human lymphocytes, a combination of a short treatment followed by a short and a long recovery, as well as a long treatment followed by a recovery was needed. Thus, in the following, for simplification, the concordance of the results will be evaluated on the basis of these combinations only.

4.3. Cytochalasin B

It has been shown previously that the use of cytochalasin B increased the sensitivity of the test in lymphocytes [20,102–104,121,166,167], because only cells which had divided are considered in the genotoxicity evaluation. It was therefore highly recommended to conduct the assay in the presence of cytochalasin B. For the cell lines, where cells are actively dividing, this recommendation may be questionable. In this study, the influence of cytochalasin B on the numbers of spontaneous micronucleated cells was therefore analysed in each cell line (Table 5 and Fig. 1). With fibroblastic cell lines, the spontaneous levels of micronucleated cells were slightly higher with cytochalasin B. But the fact that mononucleated cells without cytochalasin B are compared with binucleated cells with cytochalasin B may result in dilution of micronucleated cells in the absence of cytochalasin B and may account for this difference. However, in L5178Y cells, the incidence of spontaneous

micronucleated cells with cytochalasin B was roughly twice that seen in the absence of cytochalasin B. With mitomycin C, as the concentrations used for the positive controls were the same when using cytochalasin B or not, the possible effects of cytochalasin B were evaluated for each concentration of mitomycin C and each treatment-recovery schedule (Table 7). Induced numbers of micronucleated cells were slightly higher with cytochalasin B for all cell lines, reflecting again the apparent doubling of incidences of micronucleated cells in binucleated cells. However, as for the spontaneous incidence of micronucleated cells in fibroblastic cells, a theoretical two-fold ratio was not found. This may be due to a partial inefficiency of cytochalasin B in blocking cytokinesis. The specificity and sensitivity, and subsequent concordance, measured with the tested compounds in the cell lines, were similar in the presence or absence of cytochalasin B for each kind of compound (Table 13). No difference in the overall performances of the in vitro micronucleus test was seen in this study when using cytochalasin B or not.

4.4. Counting mononucleated and binucleated cells

Illustrative examples of the relative proportion of micronucleated mononucleated and binucleated cells were represented in Figs. 2 and 3 in L5178Y cells and human lymphocytes. This example is representative of the results obtained among the assays and the laboratories. The results obtained with bleomycin and with colchicine were chosen. Indeed, bleomycin is a typical clastogen, inducing also micronucleated mononucleated cells (see above), and colchicine is a typical aneugen known to increase the number of micronucleated mononucleated cells. We kept in mind that two mononucleated daughter-cells are the result of one cell division and that only one binucleated cell comes from the same event to compare the proportions of micronucleated mono and binucleated cells. Thus, for the comparison, we took into account that the incidence of micronucleated cells in binucleated cells must be theoretically divided by two to be compared to this incidence in mononucleated cells.

With bleomycin, the major proportion of micronucleated cells was generally found in the population of binucleated cells, irrespective of the treatment-recovery schedules, in L5178Y cells and in human lymphocytes. The distribution of micronuclei between mononucleated and binucleated cells was more specific with colchicine, as a majority of micronucleated cells was detected in mononucleated cells, taking into account that the incidence of micronucleated cells must be doubled in mononucleated cells to be compared to this incidence in binucleated cells. This was in accordance with the results previously reported [29,164]. Additionally, at high concentrations, even when the effect-concentration curve of all the micronucleated cells reached a plateau, the relative proportion of micronucleated binucleated cells tended to increase to the detriment of mononucleated cells. It was also observed in L5178Y cells (see subsequent publication on L5178Y cell line in this issue, [101] Table 1) that colchicine and griseofulvin would have been less clearly detected in the presence of cytochalasin B, after the short treatment, if only binucleated cells were taken into account for the induction of micronuclei.

In conclusion, when using cytochalasin B, counting mononucleated cells in addition to binucleated cells provides a useful indication of the mechanism of action of the tested compound for a low additional cost in terms of time and resources, and improves the accuracy of the in vitro micronucleus test. By fact, this might also improve the ability to detect a genotoxic effect even at high cytotoxic concentrations where the number of micronucleated binucleated cells tends to decline.

4.5. Cell types

The spontaneous levels of micronucleated cells were collated from the negative controls of the main studies and are illustrated by Fig. 1 and Table 5. In the absence of cytochalasin B, the backgrounds of micronucleated cells were similar in CHO and CHL cells (approx. 10–14 per 1000 cells), while in L5178Y cells, the levels of spontaneous micronucleated cells were clearly lower than in other cell lines (3–7 per 1000 cells). In the presence of cytochalasin B, the lowest backgrounds of micronucleated cells were found in human lymphocytes in primary cultures (approx. 7–9 per 1000 cells), as compared with CHO and CHL cell lines (10–14 per 1000 cells). This difference between primary cells and cell lines is not surprising and was previously reported in the in vitro chromosome aberration test [16]. However, when cytochalasin B was used, the spontaneous levels in L5178Y cell lines (approx. 8–12 per 1000 cells) were intermediate between primary cultures of human lymphocytes and, CHO and CHL cell lines. Therefore, the presence of cytochalasin B was shown to increase the levels of spontaneous micronucleated cells in the L5178Y cell line (3–7 per 1000 cells in the absence of cytochalasin B versus 8–12 per 1000 cells in the presence of cytochalasin B).

In Table 13, the ability of the different cell types used in this study to specifically detect clastogens and aneugens were compared, using the combination of short

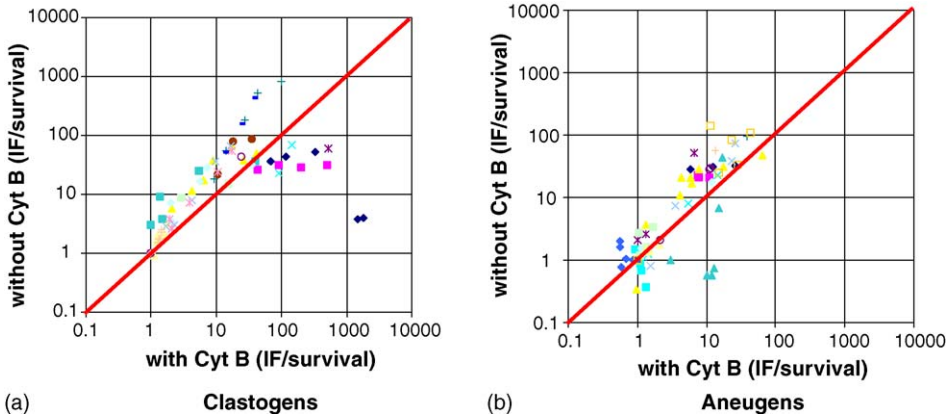

Fig. 4. Correlation with/without cytochalasin B using CHL cells: (a) for clastogens; (b) for aneugens. IF: induction factor of the incidence of micronucleated cells relative to control; each dot represents the mean of two cultures; full dots: short treatments; open dots: long treatments.

treatment and long recovery with a long treatment. The non-clastogenic compounds were identified as negative in every cell type. The human lymphocytes were found to be the least sensitive cell type (75% versus 83–100% for the cell lines), may be due to the detoxifying ability of the red blood cells present in whole blood or due to the cell-cycle dependent responsiveness of primary cultures. Moreover for this latter reason, they are expected to be more karyotypically stable and to possess normally regulated cell cycle check-points. Nevertheless, the donor variability, inherent to primary cultures of human lymphocytes, added to the inter-laboratory variability, may have affected the power of the experimental system. The cytotoxicity related to cell division slowing down or mitotic blockade seemed critical in human lymphocytes and also in L5178Y cells, but to a lesser extent. In this context, caution must be paid with base analogs and aneugens for these two cell types. In addition, following the difficulties that some participants sometimes had to obtain analysable preparations of cells, the interference of the solvent was also evaluated in the presence or absence of cytochalasin B with L5178Y. As a matter of comparison, this evaluation was also done on CHL cells (Table 6). The use of DMSO with L5178Y cells was clearly associated with the highest numbers of spontaneous micronucleated cells of the ranges, whatever the treatment-recovery used, and especially in the presence of cytochalasin B. This was not observed with CHL cells. Therefore, most attention must be paid to the use of DMSO in L5178Y in the presence of cytochalasin B.

4.6. Cytotoxicity measurements

The representation used to visualise the sensitivity for the detection of micronuclei is detailed in Section 2.13. On these graphs (Figs. 4 and 5), the higher is the IF/survival, the better is the sensitivity. For clastogens, using CHL cells or L5178Y cells, the data pooled along

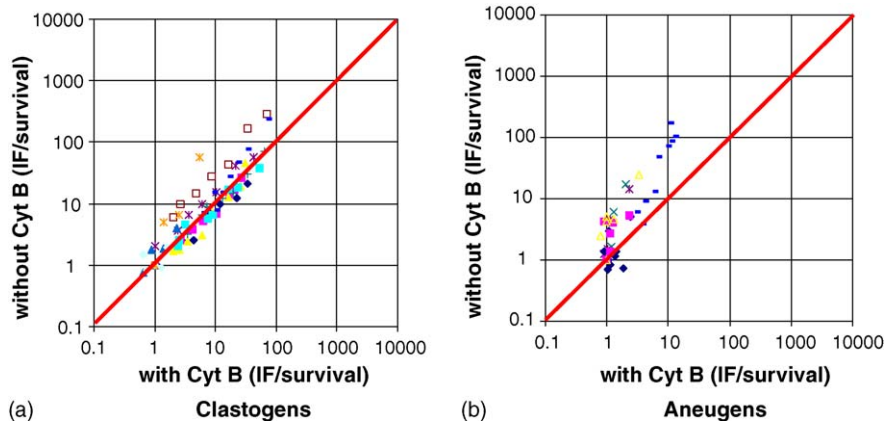

Fig. 5. Correlation with/without cytochalasin B using L5178Y cells: (a) for clastogens; (b) for aneugens. IF: induction factor of the incidence of micronucleated cells relative to control; each dot represents the mean of two cultures; full dots: short treatments; open dots: long treatments.

a bisecting line, suggesting no impact of cytochalasin B. For aneugens, the individual data gathered along a line but the majority of the data was slightly shifted above the line of equivalence. As no difference in the detection of genotoxicity was evidenced using cytochalasin B or not (see Section 4.2), this apparent difference might be due to the mode of cytotoxicity evaluation, to the viability of the total cell population considered for the percentage of binucleated cells and to the fact that the induction factors calculated from incidences of micronuclei in mononucleated cells and in binucleated cells are not strictly equivalent. However, the differences shown by these graphs, if any, were very moderate, the few outlying dots being due to extreme values of genotoxicity and cytotoxicity, likely related to experimental variability. But, comparing the incidences of micronucleated cells obtained in the presence or the absence of cytochalasin B, we must keep in mind that the methods used for evaluating cytotoxicity may give slightly different results, depending on the mechanism of cytotoxicity. The assessment of cytotoxicity needs to be improved to evaluate the genotoxic risk more accurately [168,169]. Discussions are on going as well as for the in vitro chromosome aberration test, for example on the validity of population doubling to take into account the dividing potential of the cell population [124,170].

It was interesting to note that the correlation coefficients of each experiment with the bisecting line (Fig. 5) were, for L5178Y cells for example, all above 0.75, except those corresponding to 5-fluorouracil or colchicine which were less than 0.40 (data not shown). In fact, cytotoxicities higher than 50% were often necessary to detect 5-fluorouracil and colchicine, whatever the index of cytotoxicity and short spaces between successive doses were useful in these cases. Anyway, only results obtained at cytotoxicities below 61% were considered for the interpretation of the results, taking into consideration that cytotoxicity by itself may induce irrelevant artifactual responses.

5. Conclusions

The negative compounds were found negative and well-known positive compounds were found positive. For compounds whose cytotoxicity might interfere with or make the detection of micronucleated cells difficult, such as aneugens and base analogs, caution must be paid to the level of cytotoxicity and the dose spacing. CHO, CHL and L5178Y established cell lines, as well as human lymphocytes in primary culture, were found suitable for the in vitro micronucleus assay, with less sensitivity for human lymphocytes, already known in the chromosome aberration test. For a standard protocol, we would recommend the combination of a short and a long treatment, one of them being followed by a recovery, e.g. a short treatment (3–4 h) followed by a long recovery (about 45 h) and a long continuous treatment (24 h) without recovery. There was no impact of the presence or absence of cytochalasin B on the detection of micronucleated cells on cell lines, but this might impact the cytotoxicity measurement especially for aneugens (already difficult to detect in the mouse lymphoma assay and the in vitro chromosome aberration test). Counting micronucleated cells in both mononucleated and binucleated cells when using cytochalasin B was confirmed to be useful for the detection and the identification of aneugens.

The results presented in this special issue, together with previously published data, illustrate the usefulness of the in vitro micronucleus test on CHO, CHL, L5178Y cell lines and human lymphocytes. These results clarify the most appropriate association of treatment-recovery schedules and confirm that cytochalasin B is not necessary for cell lines.

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