Short Communication

Testing Chemical Agents with the Cytokinesis-Block Micronucleus Cytome Assay

(C3A cells / genotoxicity / chemical mutagens / spindle poisons / clastogens / enzyme inhibitors)

A. MAES[1], R. ANTHONISSEN[1], L. VERSCHAEVE[1,2]

[1]Scientific Institute of Public Health, O.D. Public Health and Surveillance, Laboratory of Toxicology, Brussels, Belgium
[2]Department of Biomedical Research, University of Antwerp, Antwerp, Belgium

Abstract. In order to evaluate the applicability of the cytokinesis-block micronucleus cytome assay in routine mutagenicity testing we investigated with this method different chemicals having different mechanisms of action: non-mutagens, direct-acting base-altering mutagens, direct-acting cross-linking mutagens, clastogens including a radiomimetic chemical, indirect-acting spindle poisons and indirect-acting enzyme inhibitors. We looked at the presence of micronuclei as biomarkers for either the loss of chromosome fragments (clastogen) or the loss of a whole chromosome (aneugen), nucleoplasmic bridges as biomarkers for complex rearrangements (e.g., dicentric chromosomes) and nuclear buds as biomarkers for gene amplification. The cytome assay proved to be a suitable tool to investigate genetic effects of environmental agents and to provide insight into their working mechanisms as all chemicals tested showed the expected response.

Introduction

Cell-based assays are used extensively in toxicological investigations. This is because they can provide essential information on the potential effects of chemicals and other agents (radiation) on specific cell functions and provide a more rapid and cost-effective approach to molecular and mechanistic studies than conventional laboratory animal models. One of the important disciplines in assessing toxicity and potential adverse health effects is genotoxicity because of its well-known relationship with carcinogenesis. It is e.g. known that increased frequencies of structural chromosome aberrations and micronuclei in human peripheral blood lymphocytes are indicative of an increased cancer risk in the studied population (Bonassi et al., 1995, 2000, 2008; Hagmar et al., 1998, 2004). A couple of years ago, an extension of the 'classical' micronucleus test was presented which is now known as the cytokinesis-block micronucleus cytome (CBMN-cyt) assay (Fenech, 2006, 2007, 2009; Thomas et al., 2007). In this test (further on designated as "cytome assay"), not only the frequency of cells with micronuclei are scored, but also other morphological features that should allow a better insight into (genetic) effects of pollutants and their mode of action. Cells are scored for their viability status (necrosis, apoptosis), their mitotic status (mononucleated, binucleated, multinucleated) and their chromosomal damage or instability status [presence of micronuclei (MNi), nucleoplasmic bridges (NPBs), nuclear buds (NBUDs)]. Fluorescence *in situ* hybridization techniques, using for example whole chromosome probes, can add additional information (e.g. on aneuploidy).

So far, the CBMN-cyt assay was used for research on Alzheimer's disease (Thomas et al., 2007), the interactive effect of alcohol and folic acid on genome stability (Teo and Fenech, 2008), identification of lung cancer cases among smokers (El-Zein et al., 2008), effects of food supplements on biomarkers of cancer risk, oxidative stress and immune function (Wu et al., 2009) and radiation biodosimetry (Fenech, 2010).

Because of the anticipated importance of the cytome assay in (genetic) toxicity testing as well as in biomonitoring studies we considered it worthwhile to introduce this test in our laboratory and to evaluate its applicability in determining mechanisms by which environmental mutagens induce genetic effects. In this paper we describe the results of such a cytome test performed on a selection of chemicals belonging to different classes of mutagens, such as aneugens, clastogens (including a radiomimetic chemical), etc. The purpose was to see if these chemicals show the expected response, and hence,

Received January 4, 2012. Accepted April 2, 2012.

Corresponding author: Luc Verschaeve, Scientific Institute of Public Health, O.D. Public Health and Surveillance, Laboratory of Toxicology, J. Wytsmanstreet 14, B-1050 Brussels, Belgium. Phone: +32 2 642 54 36; Fax: +32 2 642 52 24; e-mail: luc.verschaeve@wiv-isp.be

Abbreviations: CBMN-cyt – cytokinesis-block micronucleus cytome, FISH – fluorescence *in situ* hybridization, MNi – micronuclei, NBUDs – nuclear buds, NPBs – nucleoplasmic bridges, NR – neutral red, NRU – neutral red uptake.

whether the cytome assay can be used as a tool to identify mutagens and their mode of action.

Material and Methods

The following chemicals were investigated with the cytome assay:

1) D-mannitol (Sigma, Bornem, Belgium), diethalonamide (Janssen Pharmaceutica, Beerse, Belgium) and phenanthrene (Sigma,) as non mutagenic compounds;
2) 4-nitroquinoline-oxide (4-NQO, Sigma) and methyl methane sulphonate (MMS, Sigma), which are direct--acting (base-altering) mutagens;
3) cyclophosphamide (Sigma) as a direct-acting cross--linking agent;
4) bleomycin (radiomimetic chemical, Sigma), catechol (Sigma) and $CdCl_2$ (Merck-VWR, Leuven, Belgium), which are well-known clastogens;
5) colcemid (Life Technologies, Ghent, Belgium) and vinblastine (Sigma) as indirect-acting agents and spindle poisons;
6) chloramphenicol (Sigma) and etoposide (Sigma) as indirect-acting agents and enzyme inhibitors;
7) p-nitrophenol (Fluka-Sigma) and p-eugenol (Sigma) that are usually described as *in vitro* mutagens which are ineffective *in vivo*.

Tests were performed in C3A cells (Brunschwig Chemie B.V, Amsterdam, the Netherlands) that were exposed to subtoxic doses of the chemicals for a period of 24 h according to the protocol described by Fenech (2007). The C3A hepatocyte cell line is a patented, highly selected subclone of HepG2 that retains many of the properties of the normal human hepatocyte (Kelly, 1994). It has the essential structural, biochemical, and growth features of normal human liver cells and has conserved both phase I and phase II metabolic capacities. It is therefore to be preferred over the HepG2 cell line that has partially lost its metabolizing power, or other cell lines that often have completely lost their metabolic potency. Applied doses were determined following a preliminary toxicity evaluation using the neutral red uptake test. As micronuclei (due to chromosome breakage or chromosome loss) are formed during cell division, cells were blocked in their (binucleated) telophase stage with cytochalasin B (4.5 μg/ml) to be sure that cell division occurred. Afterwards, cells were fixed and spread onto microscope slides. After staining with Giemsa-May-Grünwald, approximately 2,000 cells were investigated using a normal transmitted light microscope for the presence of micronuclei (chromosome breakage and/or aneuploidy), nuclear buds (indicating gene amplification) and nuclear bridges (dicentric chromosomes and other complex rearrangements) as described by Fenech (2006, 2007, 2009). We have found in a preliminary experiment that the vehicle controls (ethanol or DMSO) do not differ from the unexposed controls. The frequencies of aberrations (micronuclei, etc.) did indeed not change when the cells were exposed to different chemicals that were dissolved in a different vehicle, and there was also no difference between unexposed controls and vehicle controls (control cells to which the same amount and concentration of a solvent was applied as in the cells exposed to the test chemical). Therefore, our negative controls were unexposed cell cultures.

We also scored 500 cells to determine the nuclear division index which was calculated according to the following formula:

$$NDI = (M1+2M2+3M3+4M4)/N$$

With M1–M4: number of cells with 1–4 (or more) nuclei and N: total number of viable cells scored.

Here we do not report on apoptosis (programmed cell death), necrosis (cell death) and centrosome abnormalities that were not particularly induced by the subtoxic doses applied. As a matter of fact, we have seen no necrotic cells at all whatever the treatment was, and apotosis was extremely rare. Slides were coded and analysed by a scientist who was not aware of the code and hence the slides were scored without knowing to which treatment they belonged. The code was broken only when all slides were scored. The Kastenbaum & Bowman tables (Kastenbaum and Bowman, 1970) were used for determining statistically significant deviations from (unexposed) control frequencies. This binomial test was found adequate for the purpose of this investigation.

Results and Discussion

We tested several chemical mutagens in doses that were determined according to a preliminary *in vitro* toxicity test: the neutral red uptake (NRU) test. This test is based on the ability of living cells to take up and bind neutral red (NR). Neutral red is a dye which easily penetrates cell membranes via non-ionic diffusion. It accumulates in the lysosomes. Xenobiotics acting on lysosomal membranes are responsible for a decreased NRU. Living cells can therefore be distinguished from dead or dying cells based on their different NR uptake. We performed the NRU test according to well-known and validated methods as described by Repetto et al. (2008). According to this, viability curves can be established, which enables determination of the concentration that is responsible for 50 % inhibition of NRU. Concentrations used in the cytome assay were all subtoxic with the highest concentration still enabling at least 70 % viability.

Our investigation shows that all chemicals behaved as expected, according to what is known about their mechanisms of action. Table 1 gives an overview of the main results, whereas Fig. 1 shows a number of different cellular anomalies that were found in the present study.

Micronuclei data can be presented by indicating the frequency of cells with micronuclei (one or more) or as

Table 1. Results of the micronucleus/cytome assay for a few selected chemicals.

Test compound	Mechanism of action	BN	Nuclear division index (on 500 cells)	BN cells with micronuclei (‰)	BN cells with nuclear bridge (‰)	BN cells with bud(s) (‰)	Complex alterations (‰)
Negative control	-	2000	1.24	6.5	0.5	2	0.5
D-Mannitol (5 mg/ml)	-	2000	1.40	4.5	1.5	5	0
Diethalonamide (124 µg/ml)	-	2000	1.46	1.5	2	4.5	0
Phenanthrene (10 µg/ml)	-	2000	1.55	3	1	6	0
Colcemid (0.01 µg/ml)	aneugen	2000	1.46	24.5**	2	6	0
(0.025 µg/ml)		2000	1.45	29.0**	1	13.5**	0
$CdCl_2$ (73 µg/ml)	clastogen	2000	1.42	20.5**	7**	13.5**	0
(183.5 µg/ml)		450	1.35	31.1**	11.1**	51.1**	13.3**
4-NQO (0.004 µg/ml)	base-altering clastogen	2000	1.47	15**	2.5*	8**	0
(0.01 µg/ml)		2000	1.53	28.5**	2	14**	0
Cyclophosphamide (200 µg/ml)	cross-linking agent	1850	1.45	16.2**	1.6	13.5**	1.1
(500 µg/ml)		2000	1.42	32.5**	3.5*	10.5**	0.5
Chloramphenicol (250 µg/ml)	antimicrobial agent and enzyme	2000	1.49	23.5**	6.5**	16.5**	1
(625 µg/ml)	inhibitor	1000	1.38	31**	18**	53**	0
p-Nitrophenol (64 µg/ml)	+ *in vitro*/- *in vivo*	2000	1.23	14.5**	8	15.5**	0.5
(160 µg/ml)		2000	1.29	8	13*	28**	3
Negative control	-	2000	1.53	7	0	5	0
Vinblastine (6 ng/ml)	aneugen	2000	1.53	6.5	2	11.5	0
(15 ng/ml)		2000	1.54	28**	8**	21**	0
MMS (4 µg/ml)	base-altering clastogen	1346	1.57	10.4	5.2*	11.9*	0
(10 µg/ml)		2000	1.65	19**	7.5**	13.5**	0
Catechol (2.2 µg/ml)	clastogen	2000	1.39	21**	8.5**	12*	0
Bleomycin sulphate (1 µg/ml)	radiomimetic clastogen	2000	1.49	7.5	12.5**	4.5	0
(2.5 µg/ml)		2000	1.50	20**	27.5**	4	0
p-Eugenol (20 µg/ml)	+ *in vitro*/- *in vivo*	2000	1.55	9	5.5**	19.5**	0
Etoposide (0.8 µg/ml)	enzyme inhibitor	1000	1.38	18.5**	5**	11*	0
(2 µg/ml)		1000	1.32	37**	10**	22**	0.5

Results are given per 1000 analysed cells. Statistics according to Kastenbaum & Bowman tables (Kastenbaum and Bowman, 1970): * $P < 0.05$; ** $P < 0.01$). BN = number of analysed binucleated cells. Mechanisms of action as given in Kada and Ishidate (1980), Lewis et al. (1990), Tennant et al. (1990), Matsushima et al. (1999), von der Hude et al. (2000), Kirkland et al. (2008) and Katic et al. (2010).

the micronucleus frequency (number of micronuclei per 1 000 cells). In this study both are almost identical and therefore we only present the data as frequency of cells with micronuclei (‰). Experiments were performed in two series, each with its own negative control. It can be seen that the frequency of micronucleated cells in unexposed C3A cells is 6 and 7/1000 (0.6–0.7 %), respectively, which is in accordance with literature data and

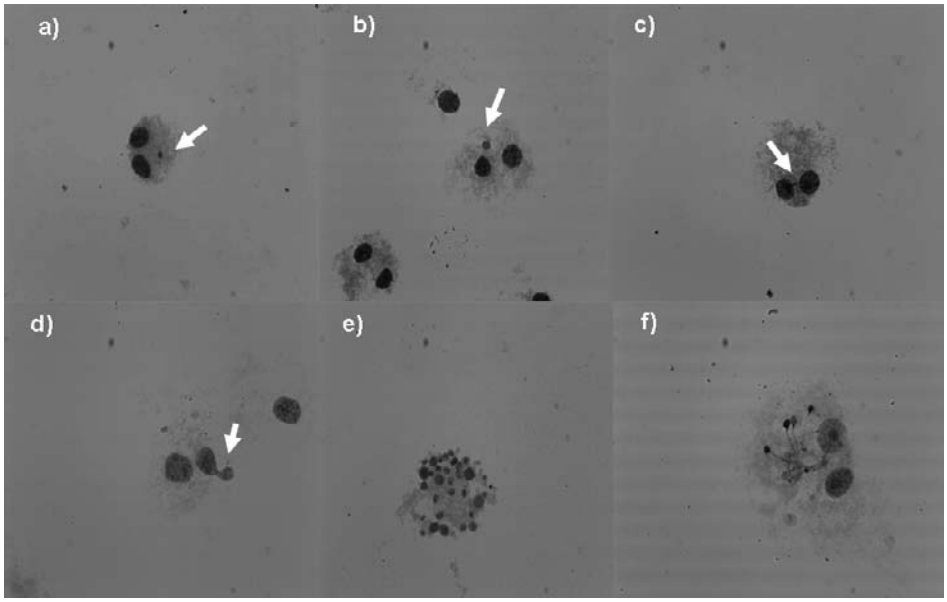

Fig. 1. Some examples of characteristic cell alterations indicating particular types of genetic effects as shown in the micronucleus/cytome assay: a) cell with small micronucleus (arrow) characteristic of a clastogenic effect; b) cell with large micronucleus (arrow) characteristic of an aneugenic effect; c) cell with nuclear bridge (arrow); d) cell with nuclear bud (arrow); e) apoptotic cell; f) cell with complex alteration appearing as multiple buds.

our own historical control values. As expected, there were only few cells with other aberrations.

D-mannitol, diethalomide and phenanthrene are non-mutagenic compounds and as expected, they produced results that were similar to these from the control cells. Bleomycin is a radiomimetic chemical, which means that its effects are more or less similar to these of ionizing radiation. The presence of micronuclei and especially nuclear bridges (which reflects chromosome rearrangements, typical for ionizing radiation) is therefore not surprising. NPBs were most prominent compared to other aberrations. Most other genotoxic compounds also significantly produced nuclear bridges, but here other aberrations prevailed. The presence of a lesser but yet significant extent of NPBs can be explained by the fact that a cell line was used instead of synchronized cells, as for example human peripheral blood lymphocytes. Cells were therefore exposed in different stages of the cell cycle and we assume that some have divided twice in culture before addition of cytochalasin B. Therefore, chromosome-type aberrations like dicentrics (and nuclear bridges) were not unexpected. The same holds true for the significant induction of nuclear buds that was seen in most of the treatments. Nuclear buds are explained as a manifestation of gene amplification resulting from DNA rearrangements (Fenech, 2006, 2007, 2009). It is anticipated that they are at the end also seen as micronuclei (Fenech and Crott, 2002).

Colcemid and vinblastine are mitotic spindle inhibitors and therefore well-known aneugens (= compounds inducing numerical chromosome aberrations). As micronuclei reflect structural as well as numerical chromosome aberrations, we expected increased micronucleus frequencies with these chemicals. Such micronuclei predominantly contain whole chromosomes, and therefore the micronuclei are larger than those that are induced by clastogens (= chromosome breaking agents). Although we didn't measure micronucleus diameters or assessed aneuploidy using other method for example fluorescence *in situ* hybridization (FISH), we indeed undoubtedly found much more larger micronuclei than with the other treatments (cf. Fig. 1b). Our results are thus in accordance with our expectations. It was, however, not the purpose of this study to fully examine the origin of micronuclei by FISH or other methods. There were also more buds compared to the controls. According to Serrano-Garcia and Montero-Montoya (2001), chromatid buds may also be the consequence of an aneugenic mechanism, i.e. due to delayed chromosome migration. The increased nuclear bud frequency may therefore also be related to the aneugenic nature of the compounds.

$CdCl_2$ is a known clastogen, and this was confirmed by the large number of cells with small micronuclei (cf. Fig. 1a). The presence of nuclear bridges indicates that whereas most chromosome aberrations were chromatid type aberrations, some chromosome type aberrations (dicentric chromosomes and/or translocations) were also induced in this unsynchronized cell line where exposures may have been over more than one cell cycle. $CdCl_2$ apparently also induces a lot of nuclear buds as 15.5 and 51.1 ‰ buds were seen at concentrations of 73 and 183.5 µg/ml, respectively. It indicates that this compound is an inducer of gene amplification as well. Table 1 also shows that there were many complex buds and alterations with a degree of complexity far above that seen with other compounds (cf. Fig. 1f). There were indeed 13.3 ‰ complex alterations (183.5 µg/ml), whereas 0 up to 1.1 ‰ only was found for the other chemicals. It should be kept in mind that these complex alterations

were found at concentrations that were subtoxic (not toxic) as indicated by the neutral red uptake test. We may thus assume that these alterations do not reflect toxicity. The significance of this is not clear at the present time but it is certainly worthwhile further investigating this compound and its mechanisms of action.

Complex alterations were more frequent at the lower dose of chloramphenicol and cyclophosphamide, but this may be without significance as frequencies were always low (max. 1.1 ‰).

The frequencies of the other aberrations were more important at the higher dose. Chloramphenicol showed induced frequencies of micronuclei as well as nuclear bridges. This is in accordance with the literature. It was indeed shown that this widely used antimicrobial agent appears to cause chromosomal effects in somatic cells, although it does not induce other genetic effects (Rosenkranz, 1988). As for $CdCl_2$, this compound is an important inducer of gene amplification as indicated by the high number of nuclear buds.

In this study we investigated only two concentrations of a compound, and therefore we can hardly speak of dose-response effects. However, it was not the purpose of the present study to establish dose-response curves but only to verify that the cytome assay is suitable to establish working mechanisms of genotoxic agents. This was clearly demonstrated. In all cases the results were in accordance with their known mode of action.

Conclusion

This investigation showed that agents with known properties regarding their genotoxic potency (or absence of genotoxicity) do show the expected response in the micronucleus/cytome assay. Some of these agents furthermore seem to induce gene amplification and sometimes exhibit complex aberrations that are less expected and merit further attention. The cytome assay proved suitable as a tool to investigate genetic effects of environmental agents and to provide insight into their working mechanisms. It is anticipated that this assay will also be useful in human biomonitoring studies, especially in synchronized lymphocyte cultures. For example, as discussed at the "Micronucleus Workshop" (EEMS conference, Florence, 2009), it appears that nuclear bridges might be a better predictor for cancer risk in a human population than the frequency of micronucleated cells or structural chromosome aberrations.

References

10th International Conference on Environmental Mutagens (ICEM) and the 39th Annual EEMS Meeting "The Renaissance of Environmental Mutagenesis" August 20–25, 2009, Florence, Italy. www.iaems.net/Historical.asp

Bonassi, S., Abbondandolo, A., Camurri, L., Dal, P. L., De, F. M., Degrassi, F., Forni, A., Lamberti, L., Lando, C., Padovani, P. (1995) Are chromosome aberrations in circulating lymphocytes predictive of future cancer onset in humans? Preliminary results of an Italian cohort study. *Cancer Genet. Cytogenet.* **79**, 133-135.

Bonassi, S., Hagmar, L., Stromberg, U., Montagud, A. H., Tinnerberg, H., Forni, A., Heikkila, P., Wanders, S., Wilhardt, P., Hansteen, I. L., Knudsen, L. E., Norppa, H. (2000) Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens. European Study Group on Cytogenetic Biomarkers and Health. *Cancer Res.* **60**, 1619-1625.

Bonassi, S., Norppa, H., Ceppi, M., Stromberg, U., Vermeulen, R., Znaor, A., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Gundy, S., Hansteen, I. L., Knudsen, L. E., Lazutka, J., Rossner, P., Sram, R. J., Boffetta, P. (2008) Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries. *Carcinogenesis* **29**, 1178-1183.

El-Zein, R. A., Fenech, M., Lopez, M. S., Spitz, M. R., Etzel, C. J. (2008) Cytokinesis-blocked micronucleus cytome assay biomarkers identify lung cancer cases amongst smokers. *Cancer Epidemiol. Biomarkers Prev.* **17**, 1111-1119.

Fenech, M., Crott, J. W. (2002) Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes – evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. *Mutat. Res.* **504**, 131-136.

Fenech, M. (2006) Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. *Mutat. Res.* **600**, 58-66.

Fenech, M. (2007) Cytokinesis-block micronucleus cytome assay. *Nat. Prot.* **2**, 1084-1104.

Fenech, M. (2009) A lifetime passion for micronucleus cytome assays – reflections from Down Under. *Mutat. Res.* **681**, 111-117.

Fenech, M. (2010) The lymphocyte cytokinesis-block micronucleus cytome assay and its application in radiation biodosimetry. *Health Phys.* **98**, 234-243.

Hagmar L., Bonassi, S., Stromberg, U., Brogge, A., Knudsen, L. E., Norppa, H., Reuterwall, C. (1998) Chromosomal aberrations in lymphocytes predict human cancer: a report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH). *Cancer Res.* **58**, 4117-4121.

Hagmar L., Stromberg, U., Bonassi, S., Hansteen, I. L., Knudsen, L. E., Lindholm, C., Norppa, H. (2004) Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts. *Cancer Res.* **64**, 2258-2263.

Kada T, Ishidate M. (eds) (1980). *Environmental Mutagens Data Book 1*, Scientist Inc., Tokyo.

Katic, J., Cemeli, E., Baumgartner, A., Laubenthal, J., Bassano, I., Stølevik, S. B., Granum, B., Namork, E., Nygaard, U. C., Løvik, M., van Leeuwen, D., Vande Loock, K., Anderson, D., Fucić, A., Decordier, I. (2010). Evaluation of the genotoxicity of 10 selected dietary/environmental compounds with the *in vitro* micronucleus cytokinesis-block assay on an interlaboratory comparison. *Food Chem. Toxicol.* **48**, 2612-2623.

Kastenbaum, M. A., Bowman, K. O. (1970). Tables for determining the statistical significance of mutation frequencies. *Mutat. Res.* **9**, 527-549.

Kelly, J. H. (1994) Permanent human hepatocyte cell line and its use in a liver assist device (LAD). US Patent Nr. 5,290,684.

Kirkland, D., Kasper, P., Müller, L., Corvi, R., Speit, G (2008) Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxic tests: a follow-up to an ECVAM workshop. *Mutat. Res.* **653**, 99-108.

Lewis, D. F. V., Ioannides, C., Parke, D. V. (1990) A prospective toxicity evaluation (COMPACT) on 40 chemicals currently being tested by the National Toxicology Program. *Mutagenesis* **5**, 433-435.

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

Repetto, G., Del Peso, A., Zurita, J. L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Prot.* **3**, 1125-1131.

Rosenkranz, H. S. (1988) Chloramphenicol: magic bullet or double-edge sword? *Mutat. Res.* **196**, 1-16.

Serrano-Garcia, L., Montero-Montoya, R. (2001) Micronuclei and chromatid buds are the result of related genotoxic events. *Environ. Mol. Mutagen.* **38**, 38-45.

Tennant, R. W., Spalding, J., Stasiewicz, S., Ashby, J. (1990) Prediction of the outcome of rodent carcinogenicity bioassays currently being conducted on 44 chemicals by the National Toxicology Program. *Mutagenesis* **5**, 3-14.

Teo, T., Fenech, M. (2008) The interactive effect of alcohol and folic acid on genome stability in human WIL2-NS cells measured using the cytokinesis-block micronucleus cytome assay. *Mutat. Res.* **657**, 32-38.

Thomas, P., Hecker, J., Faunt, J., Fenech, M. (2007) Buccal micronucleus cytome biomarkers may be associated with Alzheimer's disease. *Mutagenesis* **22**, 371-379.

von der Hude, W., Kalweit, S., Engelhardt, G., McKiernan, S., Kasper, P., Slacik-Erben, R., Miltenburger, H. G., Honarvar, N., Fahrig, R., Görlitz, B., Albertini, S., Kirchner, S., Utesch, D., Pötter-Locher, F., Stopper, H., Madle, S. (2000) In vitro micronucleus assay with Chinese hamster V79 cells – results of a collaborative study with in situ exposure to 26 chemical substances. *Mutat. Res.* **468**, 137-163.

Wu, J., Salisbury, C., Graham, R., Lyons, G., Fenech, M. (2009) Increased consumption of wheat biofortified with selenium does not modify biomarkers of cancer risk, oxidative stress, or immune function in healthy Australian males. *Environ. Mol. Mutagen.* **50**, 489-501.