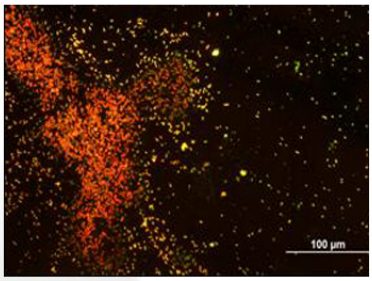
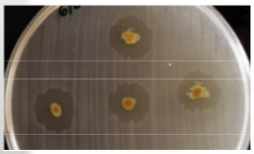
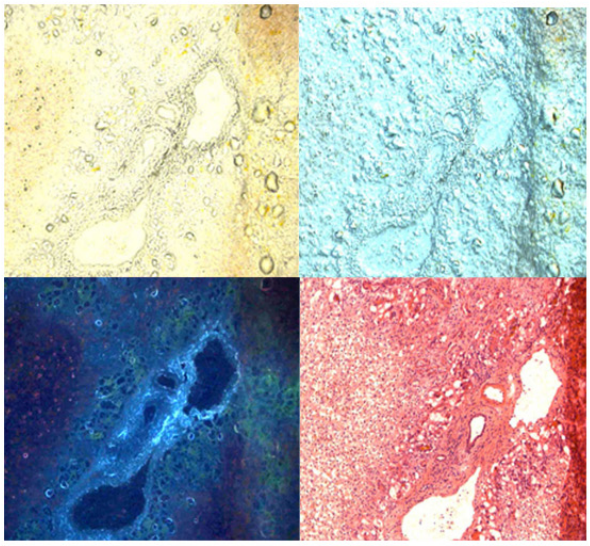
MICROSCOPY BOOK SERIES. NUMBER 4 Vol. 3







MICROSCOPY:

SCIENCE, TECHNOLOGY, APPLICATIONS AND EDUCATION

A. Méndez-Vilas, J. Díaz (Eds.)

FORMATEX research center

Microscopy: Science, Technology, Applications and Education

FORMATEX
Microscopy Series Nº 4

VOL. 3

Edited by

A. Méndez-Vilas
J. Díaz

Vol. 3
ISBN-13: 978-84-614-6191-2

FORMATEX
C/ Zurbarán 1, 2º - Oficina 1
06002 Badajoz
Spain
http://www.formatex.org
Email: micros4@formatex.org

Printed in Spain

CONTENTS

VOL. 1

VOL. 2

Techniques

VOL. 3

Applications in Physical/Chemical Sciences

Introduction to the book series

The present edition is the fourth number of a Microscopy book series, which belongs to a more general line of books published by Formatex (Badajoz, Spain), aimed at communicating current scientific and technological research in a generalistic-didactic way. In this case, the series serves to present current and emerging research topics in which microscopy plays a central role, either as the main objective of the work or as the main tool to achieve the goals, but presented to the reader in a non-specialist language and format. In this concrete area of Science/Technique, this is greatly facilitated by the intensive use of images. The series is also intended to present integrated views of the research carried out by relevant research groups across the years, rather than very punctual research results. With this aim, educationally-oriented research papers (for example, introductory chapters on a microscopy technique or an important application, using research data/results) and mini-review papers were especially called for. Mini-reviews could be thematic in nature, or focused on a specific research group activity. In the latter case, the work should provide an integrated view of the research carried out by a specific group across the years. The driving force(s) behind the evolution (seen as the reinforcement of some research approaches, refusals of others) of a certain research group can be of interest for both experts and new comers in the field, and this information is more difficult to obtain from those fragmented publications in traditional journals.

We hope that you will find the articles included in this fourth edition interesting and stimulating, and look forward to receiving new proposals for future editions.

A. Méndez Vilas, J. Díaz
Editors

The importance of microscopic analysis for accurate interpretation of chemical-induced cytotoxicity

M.J. Hazen[1], **P. Fernández Freire**[1], **J.M. Pérez Martín**[1], **A. Peropadre**[1], **O. Herrero**[1], **L. Carvajal López**[1] and **V. Labrador**[2]

[1] Cellular Toxicology Group, Department of Biology, Faculty of Sciences, Universidad Autónoma de Madrid, Darwin 2, 28049 Madrid, Spain
[2] Optical and Confocal Microscopy Service, Centro de Biología Molecular Severo Ochoa CSIC-UAM, Nicolás Cabrera 1, 28049 Madrid, Spain

The development of *in vitro* cytotoxicity assays is currently essential to evaluate the potential human and environmental health risks associated to chemical exposure, and to limit animal experimentation whenever possible. However, cytotoxicity assessments have been limited by their inability to measure morphological alterations, that can reveal subtle but extremely vital aspects of cell injury. This report presents a simple and cost-effective multi-assay approach, that includes biochemical and microscopical endpoints, to assess the underlying mechanisms involved in the toxic action of chemical compounds.

Keywords cytotoxicity, *in vitro*, cell cultures, morphological parameters

Introduction

The production of chemicals has been an important factor for the economic development of the world, but unless properly managed, their widespread use poses serious threat to the environment and human health. Since the 1930s, total production of chemicals has risen from 1 million tonnes a year to more than 400 million tonnes [1]. Consequently, the number of synthetic substances to which humans are exposed has increased dramatically over the last decades (over 100.000 now registered for use in the EU). The chemicals of major concern include pesticides and biocides, industrial compounds such as solvents, flame retardants and plastic softeners, pharmaceuticals and personal care products (PPCPs), food additives, synthetic dyes, as well as a plethora of unwanted by-products. Some of these compounds are intentionally applied to the environment and others are released, through regulated and unregulated discharges to water and air resources [2]. Hazardous chemicals can travel vast distances, causing not only an adverse impact on biodiversity at the global level, but also an increase in the incidence of some important diseases (e.g. various types of cancer, multiple allergies and lower fertility). At present, there is a lack of complete toxicological data for many environmental pollutants repeatedly found in human tissues and fluids, which makes the potential long-term consequences very difficult to estimate. Furthermore, since humans are simultaneously exposed to low doses of a large number of substances, the evaluation of the potential health hazard of chemical mixtures is a challenging toxicological problem to both the scientific and regulatory communities.

The main objectives of toxicity risk assessment are to identify adverse effects caused by exposure to chemical agents, to establish the dose-response relationships, and eventually to predict the possible public health significance. Testing methods to evaluate potential hazards of chemical compounds have been conducted over several decades in whole animal models (usually mice, rats, rabbits and dogs). However, the broad applicability of such *in vivo* studies has been often questioned because of some important interspecies differences, high-dose animal exposures and expensive and time-consuming experiments. Nowadays, considerable progress in toxicity testing strategies has been achieved as a result of development of promising technologies, public ethical concerns and current legislations. Even though animal experimentation still remains necessary, the promotion of alternative methods to characterize toxic effects potentially relevant to human health has become particularly important in recent years. A good example of this trend is the new EU legislation for Registration, Evaluation and Authorization of Chemicals (REACH), that includes specific requirements encouraging the use of alternative approaches to animal testing whenever possible [1].

Although a range of non-animal methodologies is currently available for toxicity testing [3], the efforts are now focused on selecting the most suitable models for extrapolating data obtained in experimental conditions to human risk assessment. In this context, cellular approaches are playing a key role in integrated testing strategies for chemical hazard identification, because excellent correlation has been established between *in vitro* basal cytotoxicity data and *in vivo* acute toxicity in animals and humans [4]. This observation supports the concept of basal cytotoxicity, formulated by Björn Ekwall in 1983, that most chemicals are toxic by interference with structures and/or processes essential for cell survival, proliferation and/or function [5]. Accordingly, a major goal of toxicological research is to provide new methods to evaluate chemical-induced toxicity using subcellular components, established cell lines, primary cultures, co-cultures of different cell types, tissue slices and three-dimensional cultures, preferably of human origin. Despite the broad list of *in vitro* cellular systems suitable for these studies, cell lines have been the most widely used in pharmacology and toxicology particularly because the preservation of cellular integrity, the availability from tissue

culture banks and laboratories, and the usually good standardization and reproducibility of innovative techniques for mechanistic studies.

2. *In vitro* cytotoxicity endpoints

Since a multitude of cellular events are involved in toxicity, a comprehensive *in vitro* assessement of adverse effects elicited by chemicals requires multiparametric quantitative assays, in order to mitigate the significant shortcomings of single parameter measures. In acute toxicity testing, the combined use of three main cytotoxicity endpoints which estimate metabolic activity, membrane integrity and cell number, is at present the most practical approach for evaluating basic cellular structures and function [6]. Numerous suitable methods, each one with advantages and limitations, have been developed in last years to study viability and proliferation in cultured cells. For instance, changes in metabolic activity that are considered an indicator of early cell injury can be successfully analyzed by luminescent determination of intracellular ATP levels, or by reduction of tetrazolium salts (MTT, XTT, WST-1), through both mitochondrial and cytosolic dehydrogenases, to formazan products. The assessment of plasma membrane damage, indicative of severe injury leading to cell death, is frequently achieved by means of trypan blue and fluorescent dyes which are excluded from viable cells but penetrate impaired cell membranes. Alternatively, the quantification of leakage and activity of enzymes expressed constitutively in mammalian cells, such as lactate dehydrogenase (LDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or glucose-6-phosphate dehydrogenase (G6PDH), is a useful method for detecting membrane injury in cell cultures. Another of the most standard and accurate endpoints to define basal cytotoxicity is the neutral red uptake assay (NRU), based on the ability of viable cells with intact membranes to incorporate and bind this supravital dye in lysosomes. In fact, the NRU assay on mouse BALB/c 3T3 fibroblast cell line, clone 31, is nowadays accepted by European regulations as an alternative method for phototoxicity testing [7]. On the other hand, cell number can be calculated by direct counting of viable cells after staining with a vital dye or by quantitation of total protein content, a parameter common to many applications in basic science and clinical research.

It should be noted that, despite the tendency to randomly utilize various assays, the selection of particular endpoints becomes a crucial decision that may determine the reliability of the results obtained [8]. Most of the aforementioned cytotoxicity assays can be performed in multiwell plates that permit the handling of a larger number of samples, as well as the fast evaluation of the specific endpoints using a conventional plate reader. Moreover, the information obtained from concentration-response curves is essential for extrapolating risk and safe exposure concentrations, as well as to define starting doses for subsequent *in vivo* studies. As shown in Fig. 1, relevant toxicity data such as "no observed effect concentrations" (NOEC), "lowest observed effect concentrations" (LOEC) or "half maximal effective concentrations" (EC_{50}) can be accurately determined.

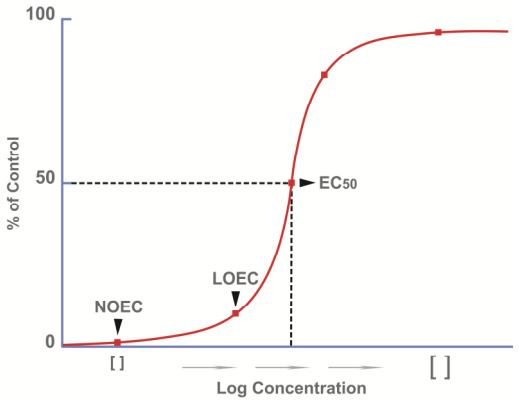


Fig. 1 A standard concentration-response curve used to plot the results of an experiment.

The quantitative *in vitro* approaches are considered as valuable predictors of human drug toxicity, although they may overlook subtle but extremely vital aspects of cell injury, involved in the mechanism of action of chemicals. When living cells are incubated with toxic substances, critical subcellular targets can undergo a sequence of structural modifications that might pass unnoticed using conventional cytotoxicity assays. In most cases, the morphologic changes become apparent only after the corresponding biochemical system has been deranged. However, chemical compounds may also interact directly with some targets within the cell (outlined in Fig. 2), inducing important alterations at the cellular and subcellular level that began to manifest almost immediately after drug exposure. Furthermore, since cell components are closely interdependent, damage to any structure and/or organelle may cause side adverse effects, which in turn eventually lead to impairment of a number of intracellular processes. Consequently, cytotoxic damage produced by chemicals is more extensively analyzed by using in parallel biochemical and morphological approaches.

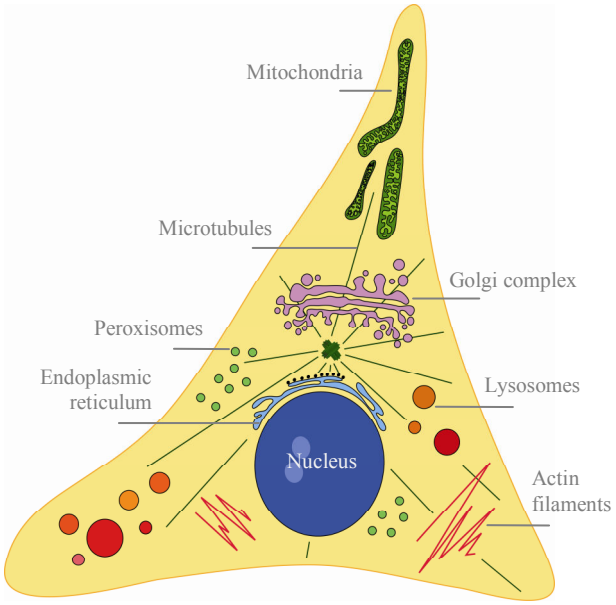


Fig. 2 Main potential subcellular targets of chemical-induced toxicity in mammalian cells.

The changes in cell integrity resulting from chemical treatments can be examined by different types of microscopy, depending on the purpose of the study. Gross modifications such as blebbing, vacuolization or differences of the cell shape in monolayer cultures, are evident on brightfield or phase contrast microscopy, whereas slight subcellular alterations are best characterized using specific fluorescence probes. Because of its intrinsic selectivity, fluorescence imaging allows the examination of cultured cells both *in vivo* or after fixation, thus becoming an important tool in cytotoxicity studies. Cellular injury can also be investigated using live cell imaging or video microscopy techniques, that are driving a revolution in both biological and clinical research. These methodologies permit the estimation of discrete steps in a sequence of events occurring at the cellular level, on different timescales ranging from seconds to hours. In cytotoxicity studies, this could be of particular importance to recognize the switch between reversible and irreversible damage, commonly referred to as the "point of no return", that defines the boundary line between cell injury and cell death. It should be noted that this type of analysis requires a careful selection of labeling techniques, to avoid impairment of cell viability during the observation period, as well as the utilization of tissue culture chambers specifically designed for the microscope stage, coupled to digital image-capturing devices [9].

In some circumstances, transmission or scanning electron microscopy studies are essential for a comprehensive understanding of cellular responses caused by chemical compounds. Some ultrastructural features of severe injury, such as osmotic swelling of mitochondria depicted in Fig. 3, can only be identified accurately by transmission electron microscopy (TEM). It must be emphasized that this drastic morphological change, that may occur as a consequence of a variety of insults including chemical exposure, is indicative of mitochondrial dysfunction and represents a decisive event to trigger apoptosis or oncosis pathways culminating in cell death. On the other hand, the use of scanning electron microscopy (SEM) is an extremely valuable tool in the analysis of cell surface alterations, such as blebbing, loss of microvilli, filopodia formation, smoothing and shrinking, showed in Fig. 4. These characteristic morphological hallmarks, thought to be a definite manifestation of irreversible damage to plasma membrane, are intimately related to toxic effects leading to imbalance of cellular homeostasis, loss of cell volume regulation and ultimately cell death.

It should be stressed that microscopical studies are not necessarily restricted to qualitative descriptions, since interesting morphometric evaluations can also be performed at the cellular level, by quantitative computer-assisted analysis of microscopic images.

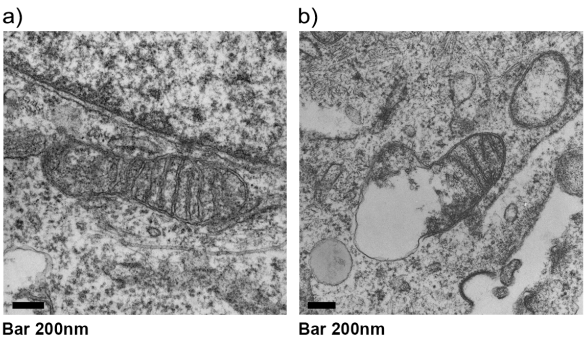


Fig. 3 TEM micrographies showing a part of the cytoplasm of a human HeLa cell. a) Intact mitochondrion next to the nuclear envelope. b) Damaged mitochondrion with pale matrix and disrupted cristae, indicating osmotic swelling.

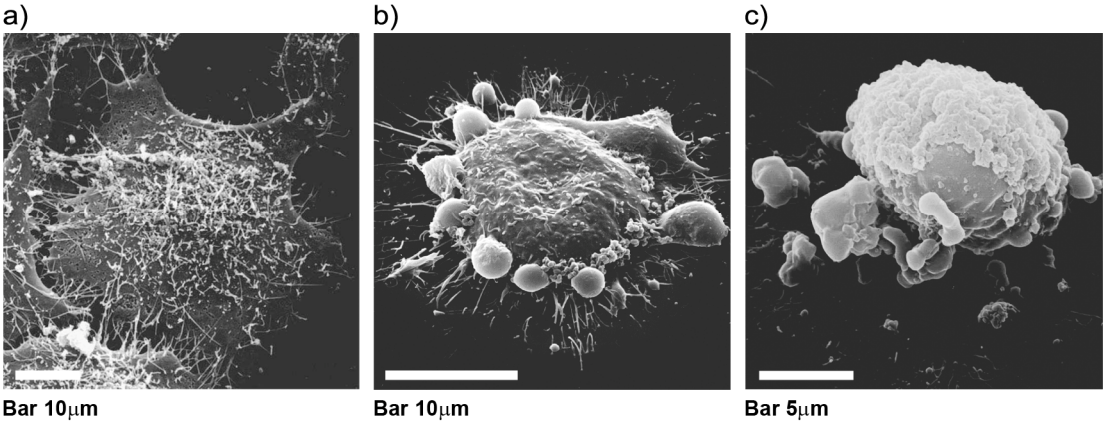


Fig. 4 HeLa cells observed by SEM microscopy revealing cell surface alterations indicative of severe injury. a) Control cell showing flat polygonal morphology and typical microvilli. b) Blebs and filopodia formation in an irreversibly damaged cell. c) Reduced adhesion, rounding and smoothing in an apoptotic cell.

3. Illustrative examples of a multi-assay approach combining biochemical and microscopical parameters

Three emerging pollutants were selected in this report to demonstrate the effectiveness of evaluating the impact of chemical exposure on mammalian cultured cells. 5H-dibenzazepine-5-carboxamide, recognized with the generic name carbamazepine (CAS No 298-46-4) is extensively used for treating epileptic seizures, and as a result one of the most frequently reported pharmaceuticals in surface water [10]. 5-chloro-2-(2,4-dichlorophenoxy)-phenol, commonly known as triclosan (CAS No 3380-34-5), is a wide spectrum biocide incorporated into many house cleaning and consumer products, which has been repeatedly found in animal tissues and human samples, as well as in the aquatic environment [11]. Pentachlorophenol (CAS No 87-86-5), used with restrictions as a general herbicide, pesticide and wood preservative, is considered nowadays as a significant water contaminant due to its slow and incomplete biodegradation [12].

The present study was performed according to an experimental protocol, that includes classical toxicological criteria (dose-response, cell number and viability analysis, morphological parameters), consistently used by our group for the toxicological evaluation of numerous chemical compounds. The methods used allow a quantitative, qualitative, and statistical approach and offer the advantages of high sensitivity, speed, reproducibility and cost effectiveness.

3.1. Experimental procedures

Vero cell line, derived from kidney of the African green monkey *Chlorocebus aethiops* (ATCC CCL-81), was selected to perform all the experimental procedures taking into consideration its suitability for both toxicological studies [13,14] and microscopic analysis [15]. Cell cultures were routinely maintained at 37ºC in a 5% CO_2 humidified atmosphere using Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (all from Lonza, Switzerland). Cells were seeded at a density of 2.5 x 10^5 cells/ml into 24-well tissue culture plates (BD FalconTM, California, USA) for quantitative analysis, or into 6-well plates (1 x 10^5 cells/ml) containing a sterile glass coverslip in each well for morphological studies, and cultured in complete medium for 24 h before adding the treatments. After removing cell culture medium and washing in phosphate buffered saline (PBS), exponentially growing cells were incubated for 24 h with increasing concentrations of carbamazepine (CBZ), triclosan (TCS) or pentachlorophenol (PCP). Working solutions of test compounds were freshly prepared by appropriately diluting the respective stock solutions and sterilized by filtration through a 0.22 μm Millipore® filter. Solvent concentration in medium was lower than 1% in all cases, including the control groups.

Colorimetric assays used to determine the basal cytotoxicity of the selected chemicals were carried out according to standardized methods, and included quantitation of total protein content (TPC) to estimate cell number [16], MTT reduction to evaluate metabolic dysfunction [17] and neutral red uptake (NRU) to assess membrane stability [18]. Absorbances were measured at the appropriate wavelengths using a Spectrafluor microplate reader (Tecan, Austria). On the other hand, statistical studies including analysis of variance (ANOVA) with Bonferroni or Games-Howell *post hoc* tests and non-linear regression for the determination of EC_{50} values, were carried out using GraphPad Prism 4.0 for windows (GraphPad Software Inc., USA). The level of statistical significance was in all cases $p \leq 0.05$.

Complementary morphological studies to evaluate the integrity of key cellular structures and organelles were conducted using different microscopic techniques. The metachromatic dye toluidine blue was employed to check the

overall morphology of Vero cells. *In vivo* fluorescent probes were used to visualize the mitochondrial reticulum (rhodamine 123 and JC-1) and the endosomal compartment (acridine orange). In addition, specific detection of actin microfilaments (phalloidin-TRITC) and indirect immunofluorescence for both microtubules (anti α-tubulin-FITC) and centromeres (anti CENP-E-TRITC) were performed. All reagents for microscopical analysis, except acridine orange (BDH, Dorset, UK) and CENP-E (gift from Dr. Suja, Universidad Autónoma de Madrid), were purchased from Sigma (USA). Microscopic observations were carried out using an Olympus BX-61 epifluorescence microscope (Tokyo, Japan), equipped with an HBO 100W mercury lamp and ultraviolet (UV, 365 nm), blue (450–490 nm) and green (546 nm) excitation filters. The images were acquired with a CCD camera Olympus DP-70 and processed using the software Olympus DP controller 1.1.1.65 and Adobe Photoshop 9.0 (Adobe Systems Inc.).

The experimental procedures were scheduled in a step-wise approach, according to our previous experience [19]. The first step entails the systematic analysis of the main cellular targets of cytotoxicity, using quantitative and qualitative endpoints. The second step involves specific assays, depending on the results previously obtained, and focuses on the determination of the cellular response elicited by the chemical compounds.

3.2. Results and Discussion

Following a 24 h exposure of Vero cells to increasing concentrations of the test compounds, all three quantitative assays performed well and led to reproducible concentration-response curves. EC_{50} values presented in Table 1 suggest that cellular membranes could be a primary target for TCS and PCP, while reduction in cell number of monolayer cultures appears to be the only toxic effect caused by CBZ at high concentrations.

Table 1 EC_{50} (μM) values for Vero cells following exposure to chemicals

	Total Protein Content	MTT Reduction	Neutral Red Uptake
CBZ	406.2	>500	>500
TCS	9.74	18.37	9.99
PCP	27	11.89	6.77

In parallel, several morphological parameters summarized in Fig. 5, were evaluated under microscopy in order to obtain additional information. Untreated Vero cells stained with toluidine blue (TB) showed a well spread cytoplasm, clearly distinguished nuclei and strongly colored nucleoli (usually 1 or 2 per nucleus). Following treatments with high concentrations of CBZ (500 μM) for 24 h, no considerable differences were observed with respect to TB-stained control cells. Conversely, cell cultures treated with 10 μM TCS exhibited intense vacuolization compatible with hydropic degeneration, with no further modifications in normal cell morphology. On the other hand, microscopic observations after treatments with PCP 40 μM showed mild cellular rounding up and retraction.

The mitochondrial reticulum was analyzed by fluorescence microscopy, under blue excitation, using the cell-permeant dye rhodamine 123 (Rho123) which selectively accumulates in mitochondria based on the membrane potential [20]. It is worth mentioning that Vero cells exhibit a characteristic perinuclear distribution of thread-like mitochondria, with a relatively low membrane potential ($\Delta\Psi_m$), even under normal conditions [21]. All the three evaluated compounds induced evident alterations in the normal appearance of mitochondria including mild fragmentation, after PCP exposure, and severe fragmentation accompanied by both swelling and $\Delta\Psi_m$ changes, following treatments with CBZ or TCS.

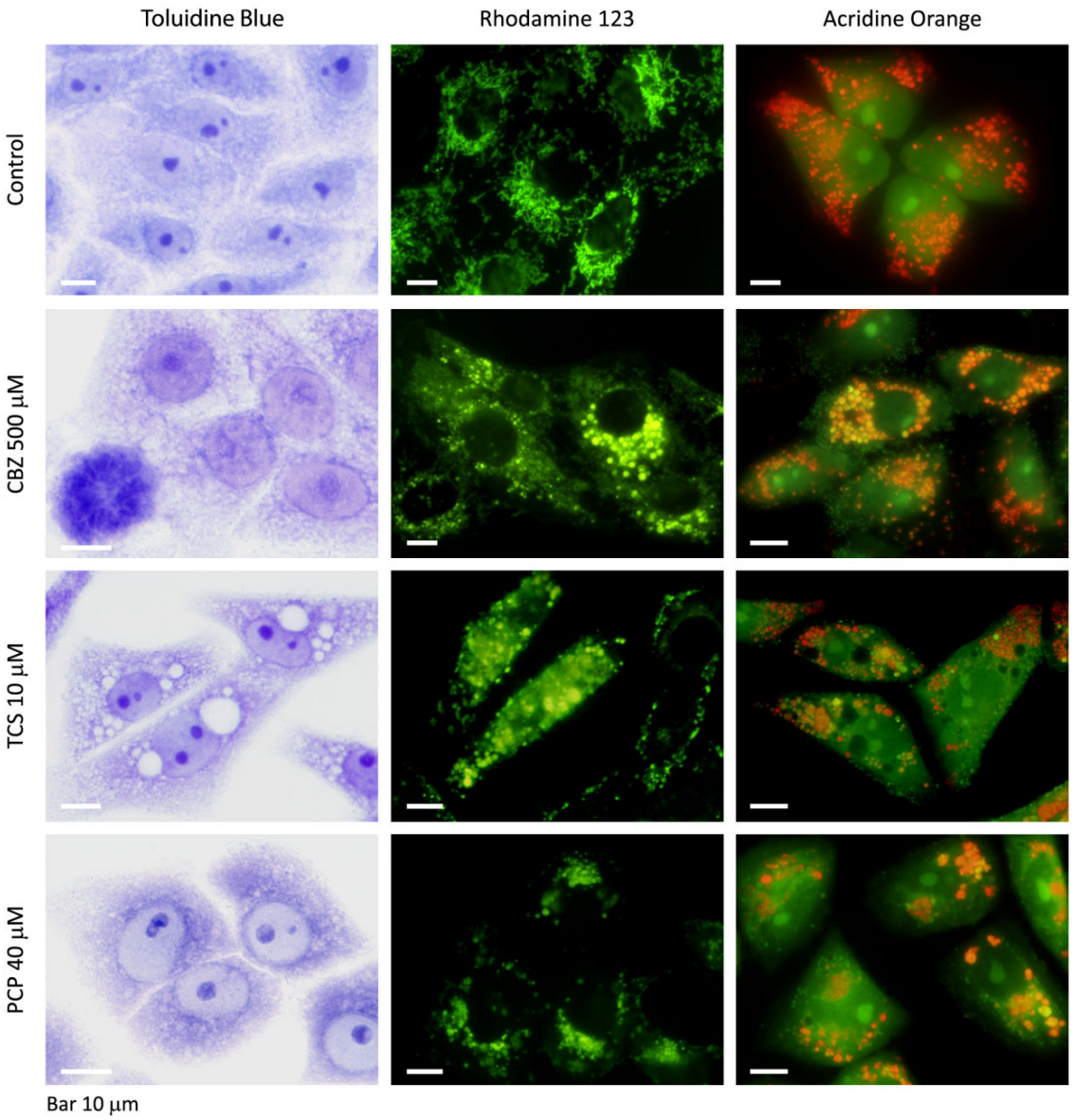


Fig. 5 Representative images of Vero cells after treatment with selected concentrations of CBZ, TCS or PCP, showing overall cell morphology after TB staining (0.025% for 1 min.), mitochondrial reticulum (6.5µg/ml Rho123 for 8 min.) and endosomal compartment (10µM AO for 8 min.). The comparison between control and treated cells was performed under identical experimental conditions, including microscope configuration.

To evaluate the endosomal compartment, cells were incubated with the weak base acridine orange (AO), which mainly accumulates in lysosomes due to proton trapping. When excited by blue light, this lysosomotropic compound shows an emission spectrum which depends on the internal pH of the acidic vesicles. Normal untreated Vero cells showed the presence of a well-defined red granular lysosomal compartment, indicative of low internal pH, concentrated in the perinuclear area. All the experimental treatments produced noticeable perturbations of endosomal system. CBZ 500 µM caused alkalinization of the acidic vesicles, shifting the fluorescence from red to yellow-orange, and mild swelling of lysosomes. After exposure to TCS 10 µM or PCP 40 µM during 24 h, "pale cells" (reduced number of intact lysosomes and/or swollen and alkalinized lysosomes) were observed, indicating a possible alteration of the lysosomal membrane permeability.

On the basis of the quantitative results and the first set of microscopical observations, a clear and expectable positive correlation was perceived in most experimental conditions. The best agreement between biochemical assays and microscopical analysis was observed for PCP treatments: NRU with AO staining, MTT reduction with Rho123 uptake and TPC with cell morphology. On the other hand, lysosomal destabilization observed in TCS-treated cells after AO staining, confirms the low EC_{50} values obtained with NRU assay, while the mitochondrial alterations detected with Rho123 are worse than expected according the results of MTT reduction test. In fact, the high EC_{50} values obtained for

MTT assay were partially due to a slight increase in mitochondrial activity around 10 µM, that correlates well with enhanced green fluorescence of some mitochondria loaded with Rho123. Finally, the mitochondrial reticulum and the lysosomal/endosomal compartment showed unpredicted alterations after CBZ treatments, since no EC_{50} values could be calculated for MTT or NRU assays.

The second step of the study, was conducted to gain deeper insight into the underlying toxic mechanisms of the tested compounds using more specific assays, based on fluorescence microscopy, that are summarized in Fig. 6.

In a previous report, we have demonstrated that the anticonvulsant carbamazepine at supratherapeutic concentrations, exerts antiproliferative effects in mammalian Vero cells [22]. This may explain the results obtained in the TPC assay, indicating a reduction of cell number in treated cultures. However, to extend these findings, we investigated possible alterations induced by CBZ in the mitotic spindle apparatus, a vulnerable target for molecules that interfere with cell cycle progression. Indirect immunofluorescent staining with antibodies against α-tubulin and the centromere protein CENP-E, and subsequent counterstaining with the DNA fluorochrome Hoechst 33258, revealed well-defined bipolar spindles with chromosomes properly aligned in the equatorial plate of control cells. Besides, centromeres localization can be seen as discrete dots scattered through the biorientated chromosomes. On the other hand, microscopic observations after exposure to CBZ 500 µM during 24 h showed the formation of defective monopolar spindles, with a microtubule array surrounded by a ring of chromosomes. In this case, a crescent and brighter CENP-E signal, indicative of a lack of attachment or insufficient tension at kinetochores, was observed. This abnormal disposition of chromosomes might be in turn responsible for faithful segregation during anaphase, leading to aberrant mitotic exit that usually triggers cell death.

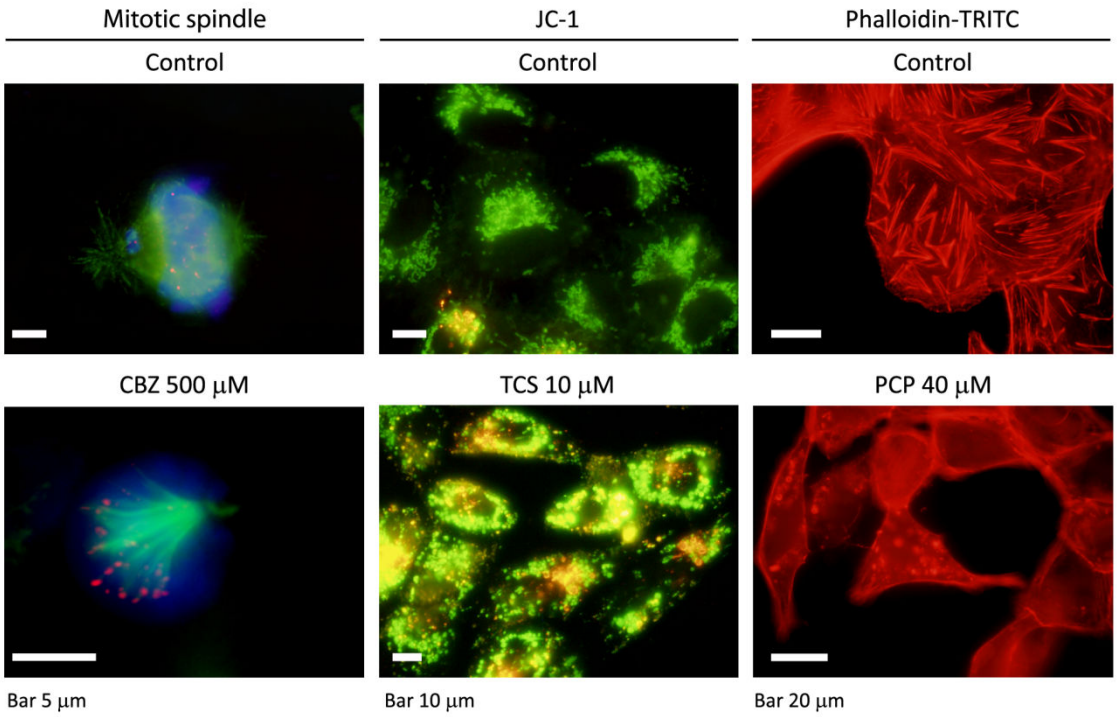


Fig. 6 Specific fluorecent probes used to identify toxic mechanisms caused in Vero cells by the tested compounds. Left panel : double-labeling immunofluorescence (α-tubulin/CENP-E) to evaluate the mitotic spindle after CBZ treatments. Middle panel : JC-1 staining for the analysis of mitochondrial ΔΨm following exposure to TCS. Right panel : Phalloidin-TRITC staining for the detection of actin cytoskeleton after PCP treatments.

One of the most striking effects observed in Vero cells treated with triclosan was the apparently inconsistent data concerning mitochondrial activity. It should be recalled that enhanced Rho123 brightness mostly reflects mitochondrial hyperpolarization (increased ΔΨm), that can be specifically detected by the dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide). In cells with high mitochondrial membrane potential, JC-1 spontaneously forms complexes known as J-aggregates with intense orange-reddish fluorescence. Conversely, in cells with low ΔΨm, the cationic lipophilic dye remains in the monomeric form, which shows only green fluorescence [23]. Accordingly, mitochondria of control Vero cells stained with JC-1 displayed a green fluorescent pattern, whereas two distinct populations of fragmented mitochondria, including a few intact green ones and a majority with yellow-orange emission, were observed following exposure to TCS 10 µM for 24 h. This observation clearly demonstrates that normal

physiology of mitochondria was impaired in the presence of TCS and supports the assumption that morphological alterations can be used as a valuable index of cytotoxicity.

The toxic effects observed in Vero cells after treatment with PCP, suggest that membrane-based structures are particulary damaged. However, complementary studies were performed with the aim to identify the cause of reduced cell number, revealed indirectly by quantitation of total protein content. Taking into account that cell spreading and adhesion to the growth substratum is extremely dependent on the preservation of the actin cytoskeleton, specific detection of microfilaments with phalloidin-TRITC was conducted. Untreated cells displayed bundles of actin "stress fibres", predominantly aligned with the major axis of the cell, that disappeared after exposure to the compound. Moreover, PCP-treated cells showed spotted fluorescence throughout the cytoplasm, which most likely indicates a shortening of microfilaments into globular aggregates, and no apparent changes in the arrangement of cortical F-actin. The disorganization of actin-based cytoskeleton correlates well with the cell retraction observed after toluidine blue staining, and may account to a certain extent for the cytotoxicity of PCP. It is important to mention that cells may round up and detach as a result of cytoskeletal derangements and then, cell density and the corresponding protein content of monolayer cultures can be significantly decreased. These observations are in agreement with our previous published results, showing that there are multiple potential targets of PCP-induced toxicity in mammalian cells [19, 24].

This work was mainly focused to describe the criteria for a simple and cost-effective testing of classical cytotoxicity endpoints, without considering more sophisticated techniques readily available to researchers in this field. The proposed multi-assay approach, although simplified with respect to the *in vivo* situations, represents an adaptable, sensitive and relatively comprehensive cellular model, for the initial assessment of toxic effects elicited by chemicals. Moreover, the versatility of the experimental design enables the constant incorporation of a diversity of relevant *in vitro* assays that could be applied to almost any cell lines.

Acknowledgements This work was supported by a grant (CTM2008-00311) from the Spanish Ministry of Science and Innovation. A. Peropadre is a recipient of a contract from the Community of Madrid, Spain (Contrato de Personal Investigador de Apoyo).

References

[1] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council REgulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/105/EC and 2000/21/EC. *Official Journal of the European Union.* 2006;No 396:1-849.

[2]Daughton C, Ternes T. Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environmental Health Perspectives.* 1999;107:907-938.

[3]Lilienblum W, Dekant W, Foth H, Gebel T, Hengsteler J, Kahl R, et al. Alternative methods to safety studies in experimental animals: role in the risk assessment of chemicals under the new European Chemicals Legislation (REACH). *Archives of Toxicology.* 2008;82:211-236.

[4]Bhogal N, Grindon C, Combes R, Balls M. Toxicity testing: creating a revolution based on new technologies. *Trends in Biotechnology.* 2005;23:299-307.

[5]Ekwall B. Screening of toxic compounds in mammalian cell cultures. *Annals of the New York Academy of Sciences.* 1983;407:64-77.

[6]Weyermann J, Lochmann D, Zimmer A. A practical note on the use of cytotoxicity assays. *International Journal of Pharmaceutics.* 2005;288:369-376.

[7]EC. *First report on the harmonization of risk assessment procedures, Part 1*. European Commission, Health and Consumer Protection Directorate-General, Brussels, 2000.

[8]Kim H, Yoon SC, Lee TY, Jeong D. Discriminative cytotoxicity assessment based on various cellular damages. *Toxicology Letters.* 2009;184:13-17.

[9]Swedlow J, Platani M. Live cell imaging using wide-field microscopy and deconvolution. *Cell Struct Funct.* 2002;27:335-341.

[10]Cunningam VL, Perino C, D´ Aco VJ, Hartmann A, Bechter R. Human health risk assessment of carbamazepine in surface waters of North America and Europe. *Regulatory Toxicology and Pharmacology* 2010;56:343-351.

[11]Singer H, Müller S, Tixier C, Pillonel L. Triclosan: occurrence and fate of a widely used biocide in the aquatic environment: field measurements in wastewater treatment plants, surface waters, and lake sediments. *Environmental Science & Technology.* 2002;36:4998-5004.

[12]ATSDR. *Toxicological Profile for Pentachlorophenol*. Altanta, GA: Agency for Toxic Substances and Disease Registry. Public Health Service, U.S. Department of Health and Human Services, 2001. p. pp. 316.

[13]ISO. *Biological Evaluation of Medical Devices. Part 5. Test for Cytotoxicity: In vitro Methods*. International Standardization Organization, 1999.

[14]Romero D, Gómez - Zapata M, Luna A, García - Fernández AJ. Morphological characterization of renal cell lines (BGM and VERO) exposed to low doses of lead nitrate. *Histology and Histopathology.* 2004;19:69-79.

[15]Grigoriev I, Chernobelskaya A, Vorobjev A. Quantitative analysis of the movements of cytoplasmic granules in polarized fibroblasts. *Membrane Cell Biology.* 1997;11:195-211.

[16]Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* 1976;72:248-254.

[17]Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods.* 1983;65:55-63.
[18]Borenfreund E, Puerner J. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicology Letters.* 1985;24:119-124.
[19]Fernández Freire P, Peropadre A, Pérez Martín JM, Herrero O, Hazen MJ. An integrated cellular model to evaluate cytotoxic effects in mammalian cell lines. *Toxicology in Vitro.* 2009;23:1553-1558.
[20]Johnson L, Walsh M, Chen L. Localization of mitochondria in living cells with rhodamine 123. *Proceedings of the National Academy of Sciences of the United States of America.* 1980;77:990-994.
[21]Diaz G, Setzu M, Zucca A, Isola R, Diana A, Murru R, et al. Subcellular heterogeinity of mitochondrial membrane potential: relationship with organelle distribution and intercellular contacts in normal, hypoxic and apoptotic cells. *Journal of Cell Science.* 1999;112:1077-1084.
[22]Pérez Martín J, Fernández Freire P, Labrador V, Hazen M. Carbamazepine induces mitotic arrest in mammalian Vero cells. *Mutation Research.* 2008;637:124-133.
[23]Smiley S, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith T, et al. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proceedings of the National Academy of Sciences of the United States of America.* 1991;88:3671-3675.
[24]Fernández Freire P, Labrador V, Pérez Martín J, Hazen M. Cytotoxic effects in mammalian Vero cells exposed to pentachlorophenol. *Toxicology.* 2005;210:37-44.