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An integrated cellular model to evaluate cytotoxic effects in mammalian cell lines

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ABSTRACT

The ever growing anthropogenic pressure to the environment has lead in 2007 to the revision of the existing legislation and the approval of the new European law regarding the production and importation of chemicals, known as REACH. This new legal framework supports the development of alternative methods to animal experimentation encouraging the improvement and/or design of new methodological strategies for the toxicological evaluation of chemical compounds.

Even though cytotoxicity studies are a reductionist approach to acute toxicity *in vivo*, they offer the best agreement between obtaining relevant information about the mechanism of toxic action and the use of alternative methods.

Following this trend, this work presents an integrated cellular strategy in order to know the toxicity and mechanism of action of chemical compounds, using simple and reproducible *in vitro* systems. The experimental procedures are performed in two steps. The first one involves the systematic analysis of the main cellular targets using proliferation, viability and morphological probes. The second step relies upon the results obtained in the first step, including specific assays that focus on the mechanism of toxic action and the cellular response.

The benefits of this strategy are exemplified with two real cases: pentachlorophenol and rotenone.

1. Introduction

The Earth is nowadays threatened by an ever growing anthropogenic pressure, mainly due to the leakage of chemical compounds to the environment. Extensive exposure to these compounds leaded to the revision of the existing legislation and a new European law, known as REACH, came into force in 2007 (EC, 2006). Due to the great quantity of toxicological studies that this situation requires, the new legal framework supports the development of alternative methods to animal experimentation according to the philosophy of the 3 Rs. This circumstance represents an exceptional chance for the improvement and/or design of new methodological strategies for the toxicological evaluation of chemical compounds (Hengsteler et al., 2006).

In this context, cell cultures turn out as a particularly interesting field, considering the wide spectrum of existing methodologies suitable for toxicological evaluation. Assuming that toxic effects seen in a whole organism are due to a prior failure of basic cellular functions (Eisenbrand et al., 2002; Walum, 1998), cytotoxicity studies offer a good compromise between using alternative methods and elucidating the mechanism of toxic action, even though they are a reductionist approach to acute toxicity *in vivo*.

According to the guidelines of international organisms (ICCVAM, 2003), this study proposes a tiered cellular approach in which proliferation, viability and morphological endpoints are combined in order to acquire fast and reproducible results, bringing together Toxicology and Cell biology and obtaining as much information as possible about the behavior of chemical compounds in mammalian cells. Two well-known environmental pollutants whose mechanism of toxic action is not fully understood (pentachlorophenol and rotenone), and three different continuous cell lines routinely used in toxicological studies (Vero, HeLa, 3T3) were selected in order to demonstrate the suitability of the experimental approach.

2. Materials and methods

2.1. Chemicals

Pentachlorophenol (PCP, CAS 87-86-5), and rotenone (ROT, CAS 83-79-4) were purchased from Sigma Chemical Co. (St. Louis, USA). Stock solutions were diluted in ethanol 100% (PCP) or dimethyl sulfoxide (ROT) and stored in darkness at room temperature.

2.2. Cell culture and treatments

Three different mammalian cell lines were selected to perform the experiments: HeLa (human adenocarcinoma), 3T3 (mouse

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embryonic fibroblasts) and Vero (monkey kidney fibroblasts). Cell cultures were routinely maintained at 37 °C in a 5% CO_2 humidified atmosphere using Dulbecco's modified Eagle's medium (DMEM), supplemented with fetal calf serum (5% for Vero and 3T3, 10% for HeLa), penicillin (100 U/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM). All reagents for cell cultures were products of Lonza (Basel, Switzerland).

Exponentially growing cells were seeded at a density of 10^5 cells/ml into 24-well tissue culture plates (BD Falcon™, California, USA) and cultured in complete medium for 24 h before adding the treatments. After washing the monolayers once in PBS, working solutions for each compound (PCP 1 – 100 μM; ROT 0.2 – 1 μM) were added by indirect dosing after sterilization by filtration through a 0.22 μm Millipore® filter. Solvent concentration in medium was lower than 0.5% (for DMSO) or 1% (for ethanol) including the control groups.

2.3. Experimental design

The experimental procedures for the toxicological evaluation were scheduled in a step-wise approach in order to gain the higher amount of information for each compound (Fig. 1).

The first step entails the systematic analysis of the main cellular targets for basal cytotoxicity by means of quantitative and qualitative assays. The battery of classical quantitative tests performed included Total Protein Content (TPC) to evaluate cell proliferation (Bradford, 1976), MTT reduction to measure mitochondrial dysfunction (Mosmann, 1983) and Neutral Red Uptake (NRU) to assess membrane stability (Borenfreund and Puerner, 1985).

Complementary morphological studies allowed us to evaluate the integrity of key cellular structures and organelles using different microscopic techniques as previously described (Labrador et al., 2007): toluidine blue staining for general morphology; *in vivo* fluorescent probes for mitochondrial reticulum (rhodamine 123) and endosomal compartment (acridine orange); specific detection of actin microfilaments (phalloidin – TRITC); and indirect immunofluorescence for the microtubule network (anti α tubulin – FITC). All the reagents but acridine orange (BDH, Dorset, UK) were purchased from Sigma.

The second step involves different experimental procedures depending on the results obtained in the first step, focusing on the determination of the mechanism of toxic action and the cellular response. The precise methods will vary depending on the results obtained for each compound in the first step (plasma membrane integrity, cell cycle progression, mitochondrial physiology, cytoskeleton integrity, endomembrane system, etc.). For the compounds tested in this study the evaluation of nuclear morphology with Hoechst 33258 (Fernández Freire et al., 2005), mitotic index scoring (Pérez Martín et al., 2008), and relocalization of acridine orange (Yuan et al., 1997); as well as specific immunofluorescence for cell death-related proteins like cytochrome c (Cyt C) and apoptotic inducing factor (AIF), performed following the instructions of the manufacturer (Santa Cruz Technologies, USA) were included.

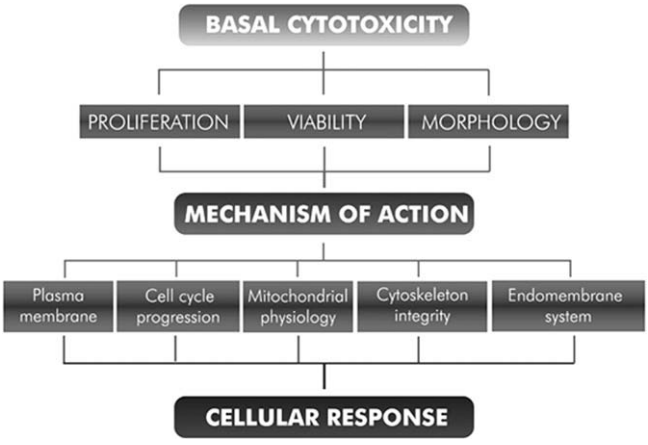

Fig. 1. Scheme of the experimental design indicating the arrangement of the different experimental procedures included.

2.4. Data analysis

Experiments were performed at least three times and each concentration group was assayed using triplicated wells. Concentration–response cytotoxicity curves were generated with individual data points expressed as percentage of that found in untreated cultures, and presented as the arithmetic mean ± standard deviation, using Microsoft® Excel 2007. Statistical analysis, including analysis of variance (ANOVA) with the appropriate *post hoc* test (Bonferroni or Games-Howell) and non-linear regression for the determination of the IC_{10} and IC_{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 $p \leqslant 0.05$.

3. Results

3.1. First step: basal cytotoxicity

3.1.1. Quantitative assays

The effect of 24 h exposure to growing concentrations of PCP and ROT on cell proliferation (TPC) and viability (MTT, NRU) of the three cell lines is shown in Fig. 2 and the IC_{10} and IC_{50} values are presented in Table 1. The toxicological pattern remains quite similar after PCP treatments independently of the cell line tested, but there are some interesting differences. Vero cells are the first ones detecting significative differences with the control in all the parameters evaluated, even though MTT assay with HeLa cells showed higher toxicity level. Despite this circumstance, concentration–response curves and IC_{10} values reveal that Vero cells were much more sensitive than HeLa and 3T3 after PCP treatments.

As far as ROT exposure is concerned, the sensitivity of Vero cell line is highlighted with all the endpoints tested, while HeLa cell line showed a mild toxicity pattern and 3T3 cell line was not severely affected.

The comparison between different endpoints in Vero cell line after PCP exposure detected significant differences from the lowest concentration tested (1 μM) only with MTT reduction test. Notwithstanding this circumstance, the IC_{50} value for NRU assay was considerably lower than those obtained with the other two endpoints.

On the other hand, concentration–response curves for ROT displayed a similar trend independently of the parameter evaluated, while the lowest IC values were achieved with the TPC assay.

On the whole, the results obtained with this quantitative assays, suggest a specific damage in the cellular membranes after PCP treatments, and alterations affecting the proliferation or adhesion of cells exposed to ROT.

3.1.2. Morphological studies

For both compounds, the first concentration with statistically significant differences (5 μM for PCP and 0.2 μM for ROT) was selected to perform the microscopic observations only on Vero cells.

Representative images of cellular organelles and structures after exposure to PCP and ROT can be seen in Fig. 3. General morphology analyzed after toluidine blue staining reveal minor

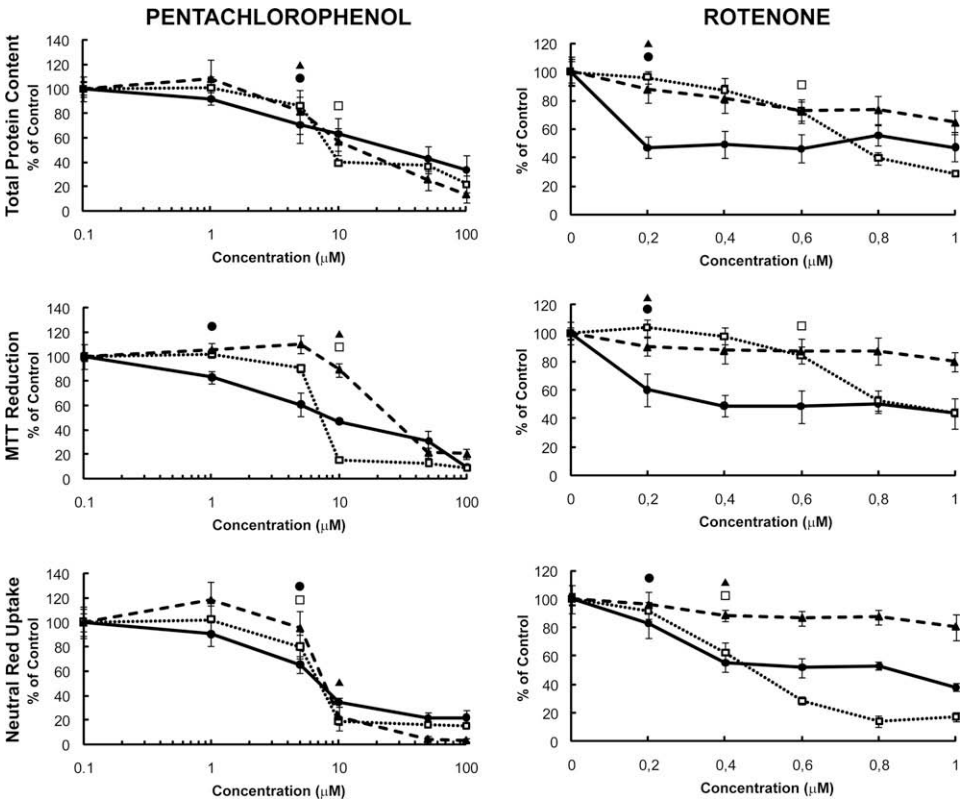

Fig. 2. Concentration–response curves after 24 h exposure of Vero (continuous line, closed circle), HeLa (dotted line, open square) and 3T3 (discontinuous line, closed triangle) cells to PCP and ROT. Statistically significance with the control ($p \leqslant 0.05$) for each cell line is represented by its own symbol.

Table 1
Comparison of EC values obtained for the three mammalian cell lines studied after PCP and ROT treatments.

Cell line	Endpoint	PCP (μM)		ROT (μM)	
		IC10	IC50	IC10	IC50
Vero	TPC	0.86	27	0.01	0.39
	MTT	0.42	11.89	0.04	0.43
	NRU	2.28	6.77	0.09	0.66
HeLa	TPC	4.61	8.25	0.42	0.73
	MTT	5.02	6.88	0.56	0.84
	NRU	4.39	6.4	0.23	0.45
3T3	TPC	3.76	11.73	0.2	>1
	MTT	9.94	11.86	0.41	>1
	NRU	5.42	7.76	0.48	>1

changes after 24 h treatment with PCP 5 μM, but severe cellular retraction and rounding up could be observed after exposure to ROT 0.2 μM. Compared with the filamentous mitochondrial network in control cells, treatment with both compounds lead to fragmentation, even though it was much more severe with PCP than with ROT. Acridine orange uptake demonstrated a mild swelling of lysosomes along with an increased green fluorescence of the cytoplasm after PCP exposure. As far as ROT exposure is concerned, no significant disturbances, apart from those derived of the cellular shrinkage, were observed after 24 h.

The integrity of the cytoskeleton was not affected by the treatments with low concentrations of PCP, with stress fibers clearly visible and no changes in the microtubule network. On the other hand, when Vero cells were subjected to ROT exposure, the integrity of the microtubules was highly compromised, even though there was no clear effect upon the actin microfilaments.

3.2. Second step: mechanism of action and cellular response

3.2.1. Pentachlorophenol

The most remarkable effect detected after the exposure of Vero cells to PCP during 24 h in the first experimental step was the disruption of the endosomal compartment, showed by quantitative (NRU) and qualitative (AO staining) endpoints. Thereby, in this second step we performed some specific experiments to determine more precisely the mechanism of action involved in the toxicity of PCP.

The AO relocalization assay is able to identify early injuries in the endosomal compartment by means of variations upon the green fluorescence of the cell. The performance of this test with a low concentration of PCP (5 μM) at three different time points (3, 4 and 6 h) is shown in Fig. 4. Even from the earliest exposure time evaluated, a significant increase in the green fluorescence intensity

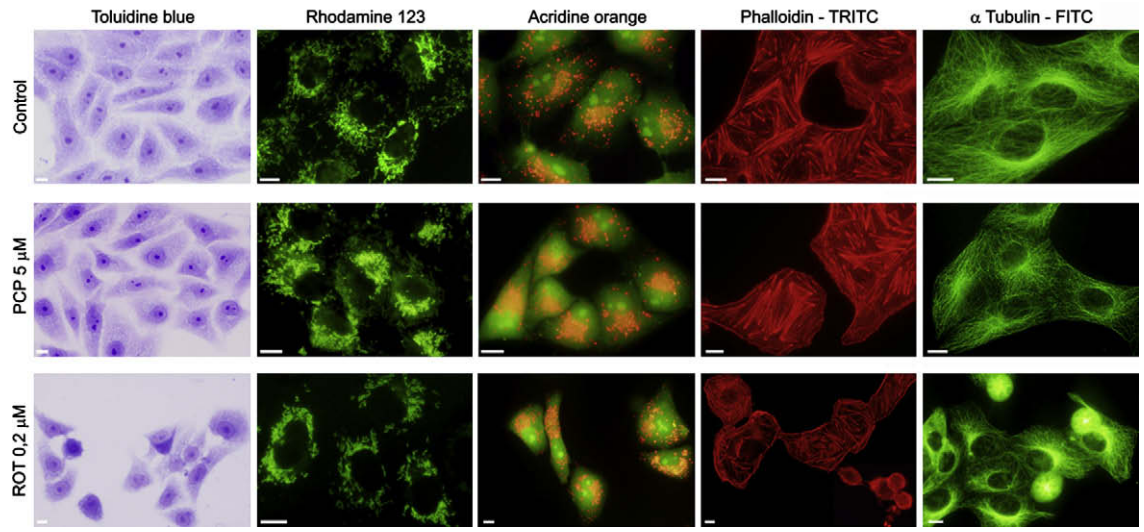

Fig. 3. Systematic analysis of the main morphological targets of Vero cells after a 24 h treatment with PCP 5 µM or ROT 0.2 µM. From left to right: general morphology, mitochondria, endosomal compartment, actin microfilaments and microtubule network. Bar = 10 µm.

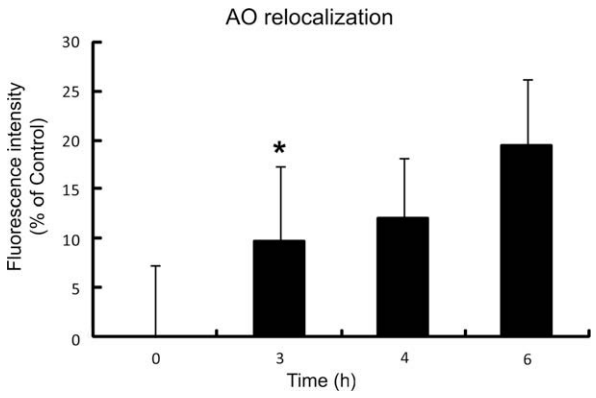

Fig. 4. Changes in the green fluorescence intensity after different time–length exposures to PCP 5 µM obtained with the AO relocalization technique. ✱ Indicates the beginning of statistically significant differences with the control.

of the cytoplasm was detected. This fact, together with the absence of mitochondrial fragmentation under these same experimental conditions, indicates an early and specific disturbance of the endosomal compartment.

In this same line, and in order to determine if the lysosomal damage was in fact affecting mitochondrial integrity, specific inmunostaining against Cyt C and AIF were performed. The distribution of AIF in control cells was congruent with mitochondrial localization. However, after exposure to PCP 5 µM during 24 h, the pattern displayed was consistent with translocation into the nucleus. Under the same experimental conditions, Cyt C remained in the mitochondria as in untreated cells (Fig. 5). This circumstance suggests a specific permeabilization of the outer mitochondrial membrane.

3.2.2. Rotenone

As the results of the first part indicates a possible interference of ROT with the proliferation and/or adhesion properties of the cultured cells, a fast and easy test as the mitotic index scoring was performed after 4, 8 and 24 h. A sudden significant increase in the division rate, due to the amount of metaphases, was detected with the shorter exposure time, reaching its maximum after 8 h (Fig. 6A). From 8 to 24 h, the number of metaphases decreased, while in parallel an equivalent number of multinucleated and dead cells was detected (Fig. 6A and B).

4. Discussion

The integrated cellular model proposed in this study has proved to be highly effective to evaluate the toxicity of chemical compounds. In the first place, the assessment of basal cytotoxicity endpoints with Vero, HeLa and 3T3 cells demonstrated the suitability of the selected battery of quantitative tests to be performed with different mammalian cell lines. In addition, this approach allowed us to compare their sensitivity to detect toxic effects. The lowest IC_{10} values obtained with Vero cells and their suitability for morphological studies signaled them as our choice to carry out all the remaining experimental procedures.

On the other hand, the advantages of a well-planed experimental design are highlighted by the interesting information acquired on each stage. The combination of proliferation and specific viability assays is a quick way to decide the primary cellular targets of a chemical compound, while complementary morphological analysis are useful either to confirm (endosomal compartment for PCP), to point out interesting side-effects (Labrador et al., 2007), and/or to show new unexpected effects (microtubule network for ROT).

Applying this experimental approach, the quantitative cytotoxic assays pointed to a preferential effect on the endosomal compartment for PCP, and to an interference of ROT either with the proliferation machinery or the adherent properties of the cells.

The disturbance of the endosomal compartment of Vero cells exposed to PCP has previously been suggested (Fernández Freire et al., 2005), but here we have been able to determine the precise underlying mechanism of toxic action. The results obtained with the highly specific acridine orange relocalization test confirmed the early disruption of the endosomal/lysosomal membranes. The partial leakage of the content of these organelles has been lately related with the initiation of the so-called lysosomal apoptotic pathway (Bidere et al., 2003), which requires the early permeabilization of the lysosomal membranes and the later disruption of the mitochondria (Kroemer et al., 2007). In this circumstances, the mitochondrial injury is mainly due to the selective permeabilization of the outer mitochondrial membrane (Galluzzi et al., 2007), which can be demonstrated, as in our case, by the release to the cytoplasm of certain apoptotic factors located in the intermembrane space

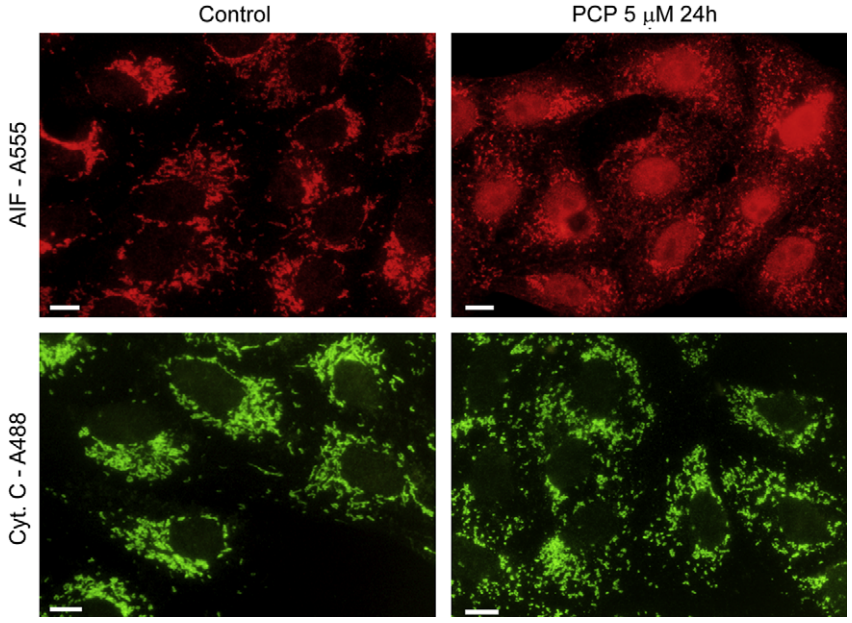

Fig. 5. Subcellular localization of death-related proteins after direct (Cyt C – Alexafluor488 1:50 overnight 4 °C) or indirect (AIF 1:200 1 h 37 °C, Alexafluor555 1:500 1 h 37 °C) immunofluorescence of Vero cells exposed to PCP 5 μM for 24 h.

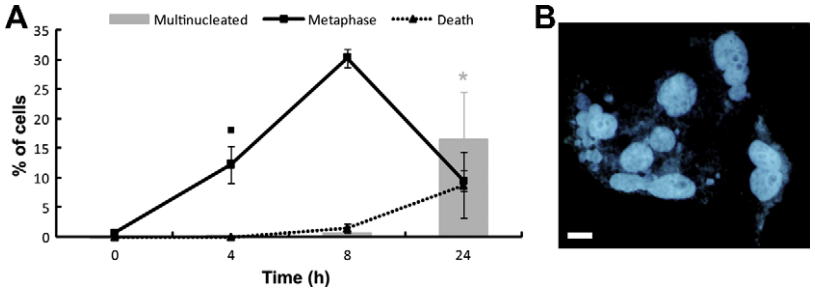

Fig. 6. (A) Percentage of metaphases, death cells, and multinucleated cells present in the cultures after different exposure times to ROT 0.2 μM. The first time point with statistically significant differences is marked with ■ (for metaphases) or ✱ (for multinucleated cells). (B) Representative image of multinucleated Vero cells (Höchst 33258 1 mg/ml 1 min) after 24 h treatment with ROT 0.2 μM. Bar = 10 μm.

(AIF), while other more tightly anchored (Cyt C), remain inside the mitochondria.

Concerning ROT toxicity, the general toxic effect detected on the first experimental step was further investigated with proliferation-related tests. The mitotic index scoring was enough to demonstrate a strong effect upon the normal cell cycle progression along time due to a severe cell cycle arrest in prometaphase, as has been previously described (Armstrong et al., 2001; Srivastava and Panda, 2007). This effect was switched onto nuclear morphology changes when the exposure time reached 24 h. The increase in multinucleated and dead cells correlated with the decrease of metaphases in this time point, which is the hallmark to identify the undergoing toxic effect as a mitotic catastrophe (Castedo et al., 2004).

On the whole, the interesting results presented in this study are a probe of the usefulness of the proposed experimental approach. The use of Vero cells for toxicological assays and the scheduled of a common set of tests, including morphological studies, followed by specific assays should be encouraged and taken into consideration for possible future validation processes.

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