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Cycloheximide inhibits the cytotoxicity of paclitaxel (Taxol®)

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Treatment of human breast (MCF-7) and lung (A549) adenocarcinoma cell lines with 10 μg/ml cycloheximide provided substantial protection from paclitaxel-induced cytotoxicity. Addition of cycloheximide to cells at 0, 6, 12 or 18 h into a 24 h exposure to paclitaxel resulted in cytotoxicity similar to that found in cells treated with paclitaxel alone for only 0, 6, 12 or 18 h, respectively. DNA flow cytometry showed that paclitaxel blocked cells in G_2/M. Mitotic index studies demonstrated that paclitaxel arrested cells in mitosis and that prolonged exposure to paclitaxel resulted in the development of multiple micronuclei. Concurrent incubation of cells in cycloheximide prevented the development of a G_2/M block, mitotic arrest and micronuclei formation. The addition of cycloheximide to cells at 6 or 12 h into a 24 h exposure to paclitaxel reduced the degree of G_2/M block to that produced by incubation of cells in paclitaxel alone for only 6 or 12 h. Mitotic index studies confirmed that cells treated with cycloheximide during paclitaxel exposure had a marked reduction in the percentage of cells in mitosis. However, the percentage of paclitaxel-treated cells which had multiple micronuclei was increased in cells treated with cycloheximide. These results indicate that entry into mitosis is a prerequisite for paclitaxel-induced cytotoxicity and that cycloheximide reduces cytotoxicity due to paclitaxel by preventing cells from entering mitosis. However, once cells have entered mitosis in the presence of paclitaxel, protein synthesis is not required for the development of multiple micronuclei and cytotoxicity.

Key words: Antagonism, cell cyle, cycloheximide, paclitaxel.

Introduction

Paclitaxel is a novel chemotherapeutic agent that is derived from the bark of the Western yew tree.[1] In clinical trials, paclitaxel has shown promising activity against ovarian[2] and breast cancers.[3] Paclitaxel also appears to be active against a number of other human malignancies, including leukemia[4] and non-small cell lung cancer.[5] In preclinical testing in mouse xenograft tumor models, paclitaxel was also active against a variety of human tumor cell lines, including melanoma and colon adenocarcinoma.[6]

Recently, we have completed a number of *in vitro* studies of paclitaxel which were designed to characterize the cytotoxicity of the drug. We have found that paclitaxel has a unique dose–response effect on human[7] cell lines. Cells exposed to paclitaxel for 24 h show a sharp decline in surviving fraction over paclitaxel concentrations of 2.5–50 nM. A plateau in cell survival is observed for cells treated with paclitaxel at concentrations of 50 nM to 1 μM. Cells exposed to very high concentrations of paclitaxel (10 μM) actually show an increase in survival which appears to be due to antagonism of paclitaxel cytotoxicity by high concentrations of Cremophor EL, the diluent in which paclitaxel is formulated for clinical use.[7,8] Although increasing the concentration of paclitaxel above 50 nM caused no additional cytotoxicity, increasing the duration of exposure of cells to paclitaxel did result in an augmentation of cell kill.

Schiff *et al.*[9] first noted the profound effect that paclitaxel has on the cell cycle. DNA flow cytometry shows that cells are blocked in G_2/M soon after exposure to paclitaxel. Mitotic index studies reveal that the majority of human tumor cells are mitotic after a 24 h exposure to paclitaxel. In addition to the cell cycle effects produced by paclitaxel, the cytotoxicity of paclitaxel is itself influenced by perturbations in the cell cycle. Cell cycle delays in G_1, produced by glutathione depletion with L-buthionine sulfoximine (L-BSO)[10] or Cremophor EL, the diluent in which paclitaxel is formulated for clinical use,[11] reduce the cytotoxicity of paclitaxel.

Cycloheximide is a potent protein synthesis inhibitor and has profound effects on the eukaryotic cell cycle.[12] Inhibition of protein synthesis occurs within minutes of exposure of cells to cycloheximide. Because of the rapid onset of action of cycloheximide and the virtually complete inhibition of cellular proliferation induced by this agent, we chose to use this agent to explore mechanisms of paclitaxel-induced cytotoxicity. We have studied the effect of cyclohex-

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imide on cell killing by paclitaxel with the use of *in vitro* clonogenic assays. Additionally, using DNA flow cytometry and mitotic index studies, we have examined the effect of cycloheximide on cell cycle perturbations induced by paclitaxel.

Materials and methods

Chemicals

Paclitaxel diluted in Cremophor EL was obtained from the Pharmacy Branch of the Clinical Center at the NIH at a stock concentration of 6 mg/ml (7.04 mM). Cycloheximide was purchased from Boehringer Mannheim (Indianapolis, IN). Propidium iodide (PI) and RNAse A were purchased from Sigma (St Louis, MO). [^{3}H]-*l*-valine was obtained from ICN Biomedicals (Irvine, CA).

Cell culture

Human breast adenocarcinoma MCF-7 and lung adenocarcinoma A549 cell lines were obtained from ATCC (Rockville, MD). They are maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. For cell survival experiments, a number of 100 mm Petri dishes were plated with 5×10^5 cells. Exponentially growing cells were exposed to paclitaxel 24 h later. Cycloheximide was added to cells at a final concentration of 10 μg/ml at various times after the initiation of paclitaxel exposure. After exposure to paclitaxel and/or cycloheximide, the cells were rinsed, trypsinized, counted, plated and incubated for macroscopic colony formation. Following incubation for 10–14 days, colonies were fixed with methanol : acetic acid (3:1), stained with crystal violet and colonies with >50 cells counted. All survival points were done in triplicate and experiments were conducted a minimum of two times. Error bars shown in the figures represent SEM and are shown when larger than the symbol.

Protein synthesis

MCF-7 and A549 cells (5×10^5) were plated into 100 mm Petri dishes. At 24 h later, paclitaxel (100 nM final concentration) or cycloheximide (10 μg/ml final concentration) or both drugs were added to cells. [^{3}H]Valine was added to cells to a final concentration of 1 μCi/ml either coincident with or at 6 or 24 h after the addition of paclitaxel and/or cycloheximide. Three hours after the addition of [^{3}H]valine, medium was removed and cells were washed five times with phosphate buffered saline (PBS; pH 7.4). Cells were removed from dishes by scraping and proteins were precipitated in 5% trichloroacetate. Cells from cultures performed in parallel which were not treated with [^{3}H]valine were collected and counted. The precipitates were collected on Whatman #3 filter papers and ^{3}H was counted in a liquid scintillation counter.

DNA flow cytometry analysis

Between 5×10^5 and 15×10^5 cells were fixed and stained in 2 ml of 0.1% sodium citrate/0.1% Triton X-100 solution containing 50 μg/ml PI. After at least 24 h in the fixative solution at 4°C, all samples were analyzed using an EPICS V cell sorter (Coulter Electronics, Hialeah, FL) with an argon ion laser tuned to 488 nm for excitation. Prior to flow analysis, 50 μg/ml of RNAse A was added to all samples. The PI fluorescence was detected using 514/530 nm filters. Both linear and log DNA histograms were collected and analyzed. For DNA degradation, log histograms are presented. For cell cycle analysis, linear DNA histograms were used and analyzed using previously described computer programs.[13]

Mitotic index

After exposure to paclitaxel and/or cycloheximide, cells were rinsed, trypsinized and washed in PBS. Cells (2.5×10^5) were briefly (<10 min) exposed to hypotonic saline (35 nM NaCl) and then fixed with methanol:acetic acid (3:1). Cells were dropped onto microscope slides and stained with crystal violet. Cells were viewed by light microscopy and the numbers of mitotic, interphase or multinucleated cells were scored. A minimum of 500 cell nuclei were scored for each time point.

Results

Protein synthesis inhibition

Paclitaxel had no effect on protein synthesis assessed by [^{3}H]valine incorporation into cells. By contrast, cycloheximide added to cells in the presence

or absence of paclitaxel reduced [^{3}H]valine incorporation by 90–95% in both cell lines (data not shown).

Cytotoxicity

As we have reported previously,[7] exposure of MCF-7 and A549 cells to paclitaxel for 24 h resulted in maximal cytotoxicity at concentrations of between 20 and 1000 nM. Concurrent incubation of the cells in 10 μg/ml cycloheximide resulted in almost complete protection from paclitaxel at all concentrations of paclitaxel (Figure 1).

To examine the time dependency of paclitaxel cytotoxicity further, cells were exposed to 100 nM paclitaxel for 6, 12, 18 or 24 h. Cells treated with paclitaxel for 24 h were then exposed to cycloheximide at 6, 12 or 18 h into paclitaxel exposure (i.e. cycloheximide exposure for the last 18, 12 or 6 h of paclitaxel exposure, respectively). Figure 2 shows that cycloheximide afforded almost complete protection from paclitaxel beginning from the time at which cells were exposed to the protein synthesis inhibitor. For example, MCF-7 cells treated with paclitaxel for 24 h had a surviving fraction of only 0.085 while the addition of cycloheximide at 6 h into the paclitaxel exposure resulted in a surviving fraction of 0.55–a degree of survival akin to the 0.64 surviving fraction seen in cells treated with paclitaxel alone for 6 h. Similar protection from paclitaxel by cycloheximide was noted at the other time points in both cell lines.

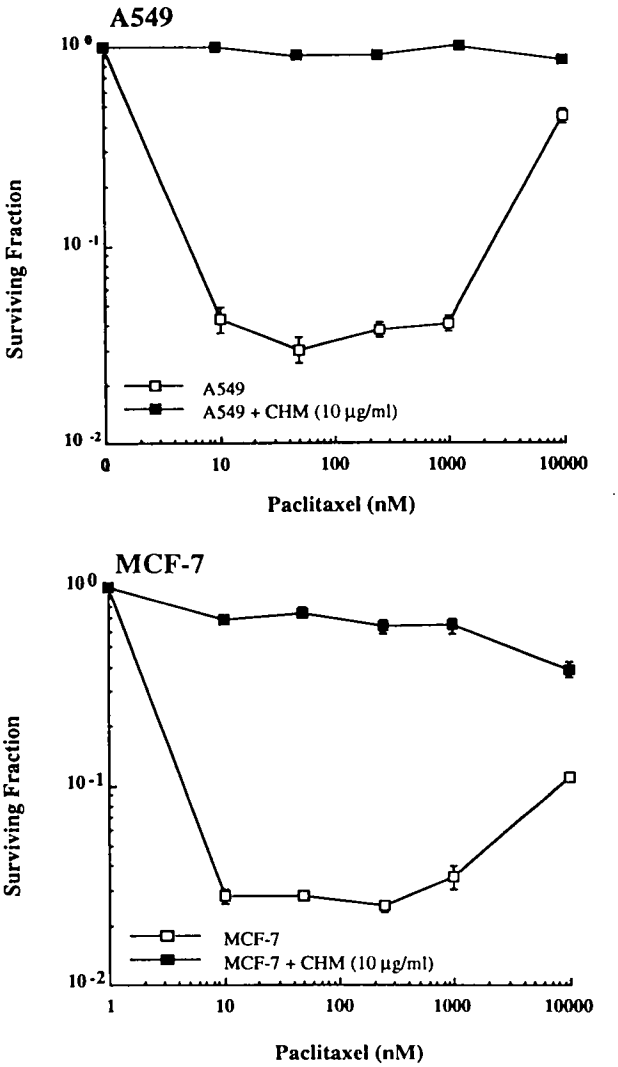

Figure 1. Survival of A549 and MCF-7 cells after exposure to paclitaxel for 24 h in the presence or absence of cycloheximide. Exponentially growing cells were exposed simultaneously to various concentrations of paclitaxel and to 10 μg/ml cycloheximide. After 24 h of exposure to the drugs, the cells were harvested and plated for clonogenic assay as described in Materials and methods. Plating efficiencies were: A549, 55%; A549 + cycloheximide, 43%; MCF-7, 76%; MCF-7 + cycloheximide, 31%.

DNA flow cytometry

We have previously shown[11] that cells exposed to paclitaxel accumulate in G_2/M in a time-dependent manner. Table 1 shows the cell cycle distribution of A549 cells exposed to 100 nM paclitaxel for 6, 12 or 24 h. Also shown in Table 1 is the cell cycle distribution of A549 cells exposed to paclitaxel for 24 h to which cycloheximide was added concurrently with paclitaxel or at 6 or 12 h into the paclitaxel exposure. After a 24 h exposure to paclitaxel, 89% of A549 cells were in G_2/M and only 1% were G_1 cells. Concurrent incubation of cells in 10 μg/ml cycloheximide, however, abolished the G_2/M cell cycle block seen after exposure to paclitaxel alone—the resulting cell populations included 44% G_1 and only 15% G_2/M cells. The addition of cycloheximide to the medium at 6 or 12 h resulted in cell cycle distributions that were similar to those seen after cells had been exposed to paclitaxel alone for 6 or 12 h. For example, 24% of A549 cells exposed to paclitaxel for 6 h were in G_1, 41% were in S and 35% were in G_2/M. When the cells were exposed to paclitaxel for 24 h but had cycloheximide added to their medium 6 h into the paclitaxel exposure, 10% were G_1, 57% were in S and 33% were in G_2/M. Similar results were obtained with MCF-7 cells.

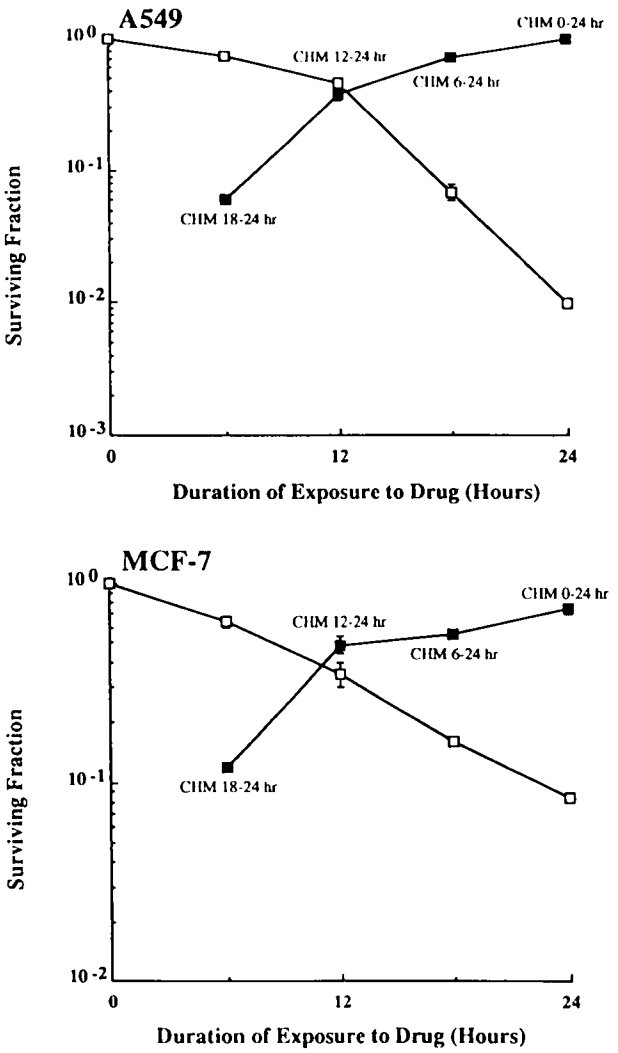

Figure 2. Survival of A549 and MCF-7 cells after exposure to paclitaxel for 6, 12, 18 or 24 h, or after exposure to paclitaxel for 24 h with the addition of cycloheximide (10 μg/ml final concentration) concurrently with or at 6, 12 or 18 h into the exposure to paclitaxel. The lines with the open squares show the survival of the cells in the absence of cycloheximide. The closed squares indicate the times at which cycloheximide was added to the cells. For the cycloheximide treated cells (closed squares), all lines are carried out to 24 h because all cycloheximide treated cells were exposed to paclitaxel for 24 h. At the completion of exposure to paclitaxel, the cells were harvested and plated for clonogenic assay as described in Materials and methods. Plating efficiencies were: A549, 59%; A549 + cycloheximide, 35%; MCF-7, 71%; MCF-7 + cycloheximide, 49%. □, paclitaxel; ■, paclitaxel (24 h) + CHM.

Mitotic index

Over time, paclitaxel produces marked changes in the mitotic index of cells. Exponentially growing human tumor cells treated with paclitaxel have an increase in the percentage of mitotic cells over a 24 h exposure to paclitaxel. After 24 h, however, the percentage of mitotic cells falls and the majority of cells develop multiple micronuclei. Table 2 shows the proportion of A549 cells in mitosis or interphase as well as the proportion of cells with multiple micronuclei. During the first 12–24 h of exposure to 50 nM paclitaxel, the proportion of mitotic cells increased sharply to between 30 and 70% of total cells. Between 24 and 48 h, however, the proportion of mitotic cells fell and the overwhelming majority of cells (>70%) were multinucleated. The addition of cycloheximide to the medium, either concurrent with or at 6, 12, 18 or 24 h into exposure to paclitaxel, greatly altered the mitotic distribution of the cells. Cycloheximide-treated cells had far fewer mitotic nuclei than did cells treated with paclitaxel alone. However, cells treated with cycloheximide did have an increase in the percentage of cells that had multiple micronuclei. In contrast to the changes in mitotic and multinucleated cells, cycloheximide had relatively little effect on the proportion of cells in interphase. These data are consistent with the concept that cycloheximide prevents the entry of cells into G_2/M. However, once cells exposed to paclitaxel are mitotic, cycloheximide cannot prevent the subsequent formation of multiple micronuclei.

Table 1. Percentage of A549 cells in various phases of the cell cycle after exposure to 100 nM paclitaxel for various times

	G_1	S	G_2/M
Control	55	38	7
Paclitaxel—6 h	24	41	35
Paclitaxel—12 h	5	36	59
Paclitaxel—24 h	1	10	89
Paclitaxel–24 h + CHM—24 h	44	41	15
Paclitaxel—24 h + CHM—18 h	10	57	33
Paclitaxel—24 h + CHM—12 h	3	38	59

Cells were exposed to paclitaxel alone or to paclitaxel for 24 h together with cycloheximide (CHM, 10 μg/ml). The cycloheximide was added to the cells either concurrently with paclitaxel (CHM—24 h), or at 6 h (CHM—18 h) or 12 h (CHM—12 h) into the paclitaxel incubation.

Table 2. Nuclear distribution of A549 cells exposed to 50 nM paclitaxel for 6, 12, 18, 24 or 48 h or to 50 nM paclitaxel and cycloheximide

Paclitaxel (50 nM)	Cycloheximide (10 μg/ml)	Interphase	Mitotics	Multinucleated
—	—	95 ± 0.5	3 ± 0.4	2 ± 0.4
6 h	—	86 ± 1	14 ± 1	0.3 ± 0.2
12 h	—	64 ± 2	32 ± 1	3 ± 0.5
18 h	—	23 ± 2	66 ± 2	10 ± 1
24 h	—	29 ± 2	62 ± 2	9 ± 1
48 h	—	6 ± 1	3 ± 0.5	92 ± 1
24 h	@ 6 h	84 ± 1	0.1 ± 0.1	16 ± 1
24 h	@ 12 h	49 ± 2	2 ± 0.5	48 ± 2
24 h	@ 18 h	18 ± 2	30 ± 2	52 ± 2
24 h	concurrent	98 ± 0.3	0.1 ± 0.05	2 ± 0.3
48 h	@ 24 h	16 ± 1	0	84 ± 1

Cells exposed to both compounds were exposed to paclitaxel for 24 or 48 h and had cycloheximide added to a final concentration of 10 μg/ml concurrently with paclitaxel or at 6, 12, 18 or 24 h into the paclitaxel exposure. Cells were harvested and prepared for mitotic index at the completion of their exposure to paclitaxel as described in Materials and methods. Figures shown are percentages of interphase, mitotic or multinucleated nuclei (±SD).

Discussion

We have shown that cycloheximide is able to protect human tumor cells from paclitaxel. Previously we have found that a prerequisite for cell killing by paclitaxel is that cells be able to enter mitosis. Cells in plateau phase of growth which would not be expected to be in cell cycle are almost completely resistant to paclitaxel.[7] Agents which induce cell cycle blocks, including L-BSO,[10] Cremophor EL,[11] doxorubicin or etoposite[14] are all able to reduce the cytotoxicity of paclitaxel. The data presented in the current study support the hypothesis that entry into mitosis is required for paclitaxel cytotoxicity. Further, the data show that cycloheximide protects cells from paclitaxel by blocking entry into mitosis.

Paclitaxel binds to and prevents the disaggregation of microtubules.[15] Although the exact mechanism of cell killing by paclitaxel is not known, cytotoxicity produced by paclitaxel is presumably largely due to paclitaxel's effects on microtubules. Cell lines resistant to paclitaxel have been described; there appear to be two common mechanisms of resistance to paclitaxel. Horwitz and coworkers originally reported the appearance of a membrane associated protein and multiple drug resistance (MDR) characteristics in the macrophage cell line J774.2 which was made resistant to paclitaxel.[16] Cabral *et al.*[17] developed Chinese hamster ovary cell lines with mutations in α- and β-tubulin that are resistant to paclitaxel and in some cases require paclitaxel for optimal growth. It is unlikely that cycloheximide antagonizes paclitaxel via a MDR-like effect. A hallmark of MDR-mediated resistance is the lack of accumulation of drugs by cells with the MDR phenotype. However, Horwitz has shown[15] and we have confirmed (data not shown) that cycloheximide does not interfere with the uptake of [^{3}H]paclitaxel by cells. Our data do not exclude the possibility that alterations in tubulin in cells exposed to cycloheximide are responsible for antagonism of paclitaxel. However, any possible alterations in tubulin produced by cycloheximide would have to occur rapidly to account for the prompt cessation in paclitaxel-mediated cytotoxicity in cells treated with cycloheximide. Finally, growth experiments show no beneficial effect of paclitaxel on the growth (or lack thereof) of cells exposed to cycloheximide. Further work is required to rigorously exclude a direct effect of cycloheximide on microtubules to account for antagonism of paclitaxel. However, any possible microtubular effect caused by cycloheximide would likely be distinct from the alterations in microtubules described by Cabral.

Cycloheximide has been shown to antagonize the cytotoxicity of a variety of chemotherapeutic agents, including actinomycin D,[18] doxorubicin,[19] etoposide[20] and vincristine.[21] A variety of mechanisms have been proposed to explain the ability of cycloheximide to prevent cell killing by chemotherapeutic drugs. These include inhibition of the depletion of essential mRNA by actinomycin,[18] inhibition of apoptosis induced by doxorubicin[19] and reduction in topoisomerase II content.[20] Of more pertinence to the current study, however, is the notion that cycloheximide can block the cytotoxicity caused by cell cycle specific agents by blocking entry of cells into a sensitive phase of the cell cycle.[21] Like the vinca alkaloids, paclitax-

el's cytotoxic activity is limited mainly to mitotic cells. By preventing cells from entering mitosis, the lethal consequences of paclitaxel exposure are also precluded by cycloheximide.

Although cycloheximide effectively blocked paclitaxel cytotoxicity when given concurrently with paclitaxel, the current study showed that protein synthesis inhibition had no effect on the progression of paclitaxel treated cells from mitosis to multinucleation. We and others[22] have noted that one effect of paclitaxel is to prolong the duration of mitosis. Using time-lapse photography, we have noted that cells treated with paclitaxel will attempt to divide but invariably cell division will be incomplete and giant cells will form with multiple micronuclei.[11] The current study shows that if cells are exposed to cycloheximide during treatment with paclitaxel, cells that are not yet mitotic will not enter mitosis implying that passage through G_2 requires active protein synthesis.[12] However, our mitotic index studies demonstrated that cycloheximide had no effect on the formation of multiple micronuclei in paclitaxel-treated cells that were already mitotic at the time of cycloheximide exposure. These data suggest that nuclear damage in mitotic cells associated with paclitaxel does not require new protein synthesis. These findings are consistent with studies that suggest that, after progression through G_2, little new protein synthesis is required by cells to complete mitosis.[23]

In summary, we have shown that cycloheximide can rapidly and completely antagonize the cytotoxicity of paclitaxel by preventing entry of cells into mitosis. However, we have also found that cycloheximide cannot protect mitotic cells from paclitaxel. Instead, mitotic cells which have been exposed to paclitaxel proceed to form multiple micronuclei even in the absence of new protein synthesis. These results are consistent with the notion that entry into mitosis is a prerequisite for the expression of cytotoxicity by paclitaxel.

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