Induction of Apoptosis and Inhibition of c-erbB-2 in Breast Cancer Cells by Flavopiridol

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ABSTRACT
Flavopiridol is a flavone that inhibits several cyclin-dependent kinases and exhibits potent growth-inhibitory activity against a number of human tumor cell lines, both in vitro and when grown as xenografts in mice. It is presently being investigated as a novel antineoplastic agent in the primary screen conducted by the Developmental Therapeutics Program, National Cancer Institute. Because breast cancer is the most common cancer and second leading cause of cancer-related deaths in women in the United States, we investigated whether flavopiridol could be an effective agent against a series of isogenic breast cancer cell lines having different levels of erbB-2 expression and differential invasion and metastatic characteristics. Flavopiridol was found to inhibit the growth of MDA-MB-435 (parental) and 435.eB (stable transfectants) cells that were established by transfecting c-erbB-2 cDNA into MDA-MB-435. Induction of apoptosis was also observed in these cell lines when treated with flavopiridol, as measured by DNA laddering, PARP, and CPP32 cleavages. We also found modest up-regulation of Bax and down-regulation of Bcl-2, but there was a significant down-regulation of c-erbB-2 in flavopiridol-treated cells. Gelatin zymography showed that flavopiridol inhibits the secretion of matrix metalloproteinase (MMP; MMPs 2 and 9) in the breast cancer cells and that the inhibition of c-erbB-2 and MMPs may be responsible for the inhibition of cell invasion observed in flavopiridol-treated cells. Collectively, these molecular effects of flavopiridol, however, were found to be independent of c-erbB-2 overexpression, suggesting that flavopiridol may be effective in all breast cancer. From these results, we conclude that flavopiridol inhibits the growth of MDA-MB-435 breast cancer cells, induces apoptosis, regulates the expression of genes, and inhibits invasion and, thus, may inhibit metastasis of breast cancer cells. These findings suggest that flavopiridol may be an effective chemotherapeutic or preventive agent against breast cancer.

INTRODUCTION
Flavopiridol [5,7-dihydroxy-8-(4-N-methyl-2-hydroxypyridyl)-6'-chloroflavone hydrochloride] is a flavone that inhibits several cyclin-dependent kinases and exhibits potent growth-inhibitory activity against a number of human tumor cell lines, both in vitro and when grown as xenografts in mice (1–5). It has attracted considerable attention because of its unique cellular targets and its ability to kill noncycling tumor cells in vitro (6). Flavopiridol is presently being evaluated in a phase I clinical trial at the National Cancer Institute (7). However, the molecular mechanism by which flavopiridol exerts its tumor suppressive effect has not been fully evaluated.

Apoptosis is one of the important pathways through which chemotherapeutic agents inhibit the growth of cancer cells. Thus, it is important to investigate whether the induction of apoptosis and alterations of apoptosis-related gene expression are associated with the molecular mechanism by which flavopiridol may exert its biological effects on breast cancer cells. The induction of apoptosis is partly mediated intracellularly by several genes, such as Bcl-2 and Bax (8). Bcl-2 functions as a suppressor of apoptotic death triggered by a variety of signals (9), whereas a predominance of Bax over Bcl-2 accelerates apoptosis upon apoptotic stimuli (10).

Breast cancer is the most common cancer in women in the United States, and it remains the second leading cause of cancer-related female deaths in this country (11). Several genes have been implicated in breast cancer aggressiveness. c-erbB-2 is a key molecule for breast cancer metastasis. Overexpression of the c-erbB-2 gene has been found in ~20-30% of human breast cancers and has been positively correlated with invasion and metastasis in cancers (12, 13). It has been found that transfection of c-erbB-2 into breast cancer cells increased their ability for invasion and metastasis and that an increase of MMPs was detected in c-erbB-2 transfected cells (14). MMPs are believed to be key molecules for cancer invasion and metastasis (15–17). Furthermore, overexpression of c-erbB-2 has been shown to be correlated with an increase in MMP secretion and metastatic potential of breast cancer cells in experimental metastasis assays (14).

In this study, we investigated whether flavopiridol could inhibit the growth of breast cancer cell lines, MDA-MB-435 and 435.eBs (established by transfecting c-erbB-2 cDNA into MDA-MB-435), and whether c-erbB-2 overexpression may affect the sensitivities of these cells to flavopiridol. In addition, we were interested in elucidating the molecular mechanism by which flavopiridol may induce apoptotic cell death in these isogenic cell lines. We also investigated the effect of flavopiridol...
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dol on MMP\(^3\) secretion and invasion, which was previously shown to be affected by c-erbB-2 transfection. Our data show that flavopiridol inhibits the growth of breast cancer cells irrespective of c-erbB-2 overexpression, induces apoptosis, regulates the expression of genes, and inhibits invasion and, thus, may inhibit metastasis of breast cancer cells.

**MATERIALS AND METHODS**

**Cell Lines and Culture.** Human breast cancer cell line MDA-MB-435, the 435.eB transfected cell lines (eB1 and eB4), and the control 435.neo cell line were kindly provided by Dr. Dihua Yu at the University of Texas M. D. Anderson Cancer Center. All of the cells were cultured in DMEM/F12 medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS, 1% penicillin/streptomycin in a 5% CO\(_2\) atmosphere at 37°C. The 435.eB transfectants were generated by transfection of the pCMVerbB-2 plasmid containing the 4.4-kb full-length human normal c-erbB-2 cDNA and the pSV2-neo plasmid carrying the neomycin-resistance selection marker gene into MDA-MB-435 cells (14, 18). 435.eB1 and 435.eB4 cells express 258-fold and 165-fold c-erbB-2 compared to parental MDA-MB-435 (14, 18). The control 435.neo cell line was established by transfecting the pSV2-neo plasmid alone into MDA-MB-435 cells (14, 18).

**Cell Growth Inhibition.** The MDA-MB-435 cells, 435.neo cells, and 435.eB cells were seeded at a density of 5 \(\times\) \(10^5\)/well in a six-well culture dish. After 24 h, the cells were treated with 70 nM, 150 nM, and 300 nM of flavopiridol or DMSO (vehicle control). Cells treated with flavopiridol or DMSO for 1–3 days were harvested by trypsinization, stained with 0.4% trypan blue, and counted using a hemocytometer.

**Protein Extraction and Western Blot Analysis.** The breast cancer cells (MDA-MB-435, 435.neo, and 435.eB) were plated and cultured in complete medium and allowed to attach for 24 h followed by the addition of 150 nM or 300 nM flavopiridol and incubation for 24, 48, and 72 h. Control cells were incubated in the medium with DMSO using the same time points. After incubation, the cells were harvested by scraping the cells from culture dishes using a scraper and collected by centrifugation. Cells were resuspended in Tris-Cl buffer, sonicated for 2 \(\times\) 10 s, and lysed using an equal volume of 4% SDS. Protein concentration was then measured using protein assay reagents (Pierce, Rockford, IL). Cell extracts were boiled for 10 min and chilled on ice, subjected to 14 or 10% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. Each membrane was incubated with monoclonal Bcl-2, c-erbB-2 (1:500, Oncogene, Cambridge, MA), Bax (1:5000; Biomol, Plymouth Meeting, PA), and rabbit polyclonal \(\beta\)-actin (1:5000, Sigma, MO) antibodies, washed with Tween 20 in Tris-buffered saline, and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce).

**Densitometric Analysis for c-erbB-2 Expression.** Autoradiograms of the Western blots for c-erbB-2 and actin protein expression were scanned with the Chemi Doc 1000 image scanner (Bio-Rad, Hercules, CA). The bidimensional optical densities of c-erbB-2 and actin proteins on the films were quantified and analyzed with Molecular Analyst software (Bio-Rad). The ratios of c-erbB-2/actin were calculated by standardizing the ratios of each control to the unit value.

**Zymography of MMP Activity.** Gelatin zymography was performed according to the methods published previously (14, 19), with a slight modification. The breast cancer cells (MDA-MB-435, 435.neo, and 435.eB) were plated and cultured in complete medium and allowed to attach overnight. The cells were washed three times with DMEM/F12 and incubated in conditioned medium for 24, 48, and 72 h. The conditioned media were DMEM/F12 without serum and phenol red and with 150 or 300 nM flavopiridol or DMSO. The culture supernatants were collected, and cell debris was spun off. The supernatants were concentrated using spin columns (Amicon, Beverly, MA), and the protein concentration was measured using protein assay reagents (Pierce). The samples with equal proteins were then subjected to 10% nonreducing SDS-PAGE (containing 0.1% gelatin). After electrophoresis, the gels were washed with 2.5% Triton X-100 for 30 min and with developing buffer (10 mM Tris base, 40 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl\(_2\), and 0.02% Brij 35) for 1 h. After washing, the gels were incubated with fresh developing buffer overnight at 37°C. The gels were then stained with Coomassie solution (0.5% Coomassie blue in 10% methanol, 5% acetic acid) for 1 h and de-stained with same solution without Coomassie blue.

**DNA Ladder Formation.** Cellular cytoplasmic DNA from cells treated with 300 nM flavopiridol for 24, 48, 72 h or with DMSO for 24 h (as control) was extracted using 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.2% Triton X-100. The lysate was centrifuged for 15 min at 13,000 \(\times\) g to separate the fragment DNA (soluble) from intact chromatin (nuclear pellet). The supernatant from the lysate was treated with RNase, followed by SDS-Proteinase K digestion, phenol chloroform extraction, and isopropanol precipitation. DNA was separated through a 1.5% agarose gel. After electrophoresis, gels were stained with ethidium bromide, and the DNA was visualized by UV light.

**Analysis of Cleavage of CPP32 and PARP.** Cells treated with 100 nM flavopiridol or with DMSO (as control) for 24 and 48 h were lysed in lysis buffer [10 mM Tris-HCl (pH 7.1), 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 \(\mu\)M sodium orthovanadate, 2 mM iodoacetamide, 5 \(\mu\)M ZnCl\(_2\), 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X100]. The lysates were kept on ice for 30 min and vigorously vortexed before centrifugation at 12,500 \(\times\) g for 20 min. Fifty micrograms of total proteins were resolved on 14% or 10% SDS-PAGE and then transferred to nitrocellulose membrane. The membrane was incubated with primary monoclonal antiCPP32 antibody (1:250, Santa Cruz, CA) or PARP antibody (1:500; Biomol), washed with TTBS, and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce).

**Cell Invasion Assay.** The invasion assay was carried out following standard methods using the matrigel invasion assay procedure as described below. Matrigel was purchased from Dr. Dihua Yu at the University of Texas M. D. Anderson Cancer Research Center.
Becton Dickinson Labware (Bedford, MA), and Costar transwell culture inserts were purchased from Fisher scientific (Itasca, IL). The transwell was coated with 100 μl of matrigel, which were diluted in ice cold DMEM at a final concentration of 400 μg/ml and incubated at 37°C for 3 h to allow the matrigel to polymerize. DMEM was allowed to evaporate under sterile conditions under the culture hood. The matrigel-coated inserts were rehydrated by adding 100 μl of serum-free medium and incubating for 2 h at 37°C. The breast cancer cells were cultured as described earlier, trypsinized, collected by centrifugation, and resuspended in serum-free medium, and the cell numbers were calculated by using the trypan blue exclusion method using the hemocytometer. A different number of cells in 0.5 ml was dispensed into each culture inserts, and the cells were incubated in a tissue culture incubator for 18 h without and with 150 and 300 nM flavopiridol. After the incubation, the medium from the culture inserts was discarded, and the inner surface was wiped with cotton wipes to remove any noninvading cells through the matrigel. The inserts were fixed for 3 min in Hema 3 fixative, then consecutively stained with Hema 3/Eosin and Hema 3/methylene blue obtained from Fisher Scientific (Itasca, IL). The inserts were washed in distilled water, allowed to air dry, and then examined under a light microscope counting four random fields from each insert. The percent inhibition of cell invasion relative to controls was plotted using graph pad prism software.

RESULTS

Effects of Flavopiridol on Cell Growth. The effect of flavopiridol on the cell growth of MDA-MB-435 and 435 transfectants is depicted in Fig. 1. The treatment of MDA-MB-435, 435.neo, 435.eB1, and 435.eB4 breast cancer cells for 1–3 days with 70 nM, 150 nM, and 300 nM of flavopiridol resulted in inhibition of cell proliferation, which was dose-dependent. However, the dose-dependent growth inhibition was not influenced by the status of c-erbB-2 overexpression (IC50s for all three cell lines was about 70 nM). The data on the inhibition of cell growth in parental and 435.neo was found to be identical; hence, Fig. 1 represents parental cells as the control. Furthermore, in all our subsequent experiments, we could not find any difference between the parental cell line and 435.neo; therefore, all subsequent data have been presented with 435 cells as the control, except Fig. 6, in which 435.neo has been presented. The overall inhibition of cell proliferation could be due to the induction of apoptosis elicited by flavopiridol. We, therefore, investigated whether flavopiridol could induce apoptosis in these breast cancer cells.

Induction of Apoptosis. Apoptosis was demonstrated in all of the cell lines treated with 300 nM flavopiridol by the DNA ladder shown in Fig. 2A. The induction of apoptosis was pronounced at 24 h of treatment, and it is directly correlated with the inhibition of cell growth. Western blot analysis revealed that inactive CPP32 (32 kDa) was cleaved to yield an active fragment (17 kDa) after flavopiridol treatment for 24 h (Fig. 2B). Furthermore, PARP cleavage analysis showed that the full-size PARP (116 kDa) protein was also cleaved to yield an 85-kDa fragment after treatment with flavopiridol for 24 h (Fig. 2B) in all cell lines tested, corresponding with the activation of CPP32. These three independent methods of measuring apoptosis provided strong evidence that apoptosis was induced in all cell lines treated with flavopiridol and that the apoptosis inducing activity of flavopiridol was found to be independent of c-erbB-2 overexpression. To explore the mechanisms by which flavopiridol...
induces apoptosis, we investigated the alterations in the expression of genes, which are known to be involved in the apoptotic pathway.

**Expression of Bcl-2 and Bax.** The effect of flavopiridol on Bcl-2 and Bax expression in MDA-MB-435, 435.neo, and 435.eB cells were studied by Western blot analysis. The levels of Bcl-2 expression in all 435 cell lines were found to be modestly down-regulated with the addition of flavopiridol when exposed for 24–72 h (Fig. 3A). Furthermore, the expression of Bax was also found to be modestly up-regulated after 24 h of 150 nM or 300 nM flavopiridol treatment (Fig. 3B). We could not find any substantial difference in Bcl-2 and Bax expression among these 435 cell lines, suggesting that the modulation in Bax and Bcl-2 by flavopiridol is not dependent on c-erbB-2 expression.

**Expression of c-erbB-2.** To explore the effect of flavopiridol on c-erbB-2 that has been correlated with metastatic potential, the expression of c-erbB-2 in MDA-MB-435 and 435 eB transfectants with and without flavopiridol treatment was tested by Western blot analysis. As Tan et al. (14) reported, there were higher expressions of c-erbB-2 in 435.eB1 cells and 435.eB4 cells than in parental MDA-MB-435 and 435.neo cells. The expression of c-erbB-2 protein in MDA-MB-435 and 435.eB cells treated with 150 nM or 300 nM flavopiridol was significantly down-regulated at various degrees (Fig. 4A); however, the down-regulation was much more pronounced in parental compared to those overexpressing c-erbB-2. It is important to note that to detect erbB-2 expression, the blots with parental cells were exposed for a longer period of time as compared to those with overexpressing erbB-2.
compared to those with erbB-2 overexpression. To obtain a quantitative value for the protein expression of c-erbB-2, absorbance measurement was conducted as described under “Materials and Methods.” The ratios of c-erbB-2 to actin protein expression revealed that cells treated with flavopiridol showed a 55–90% decrease in c-erbB-2 compared to the untreated control (Fig. 5).

Enzyme Activities of MMPs. The activities of the basement membrane degrading MMPs in MDA-MB-435 and 435.eB transfectants with or without flavopiridol treatment were measured by zymographic analysis. Significantly higher levels of MMP-2 (72 kDa) and MMP-9 (92 kDa) were detected in the conditioned medium of the 435.eB1 and 435.eB4 transfectants than in parental MDA-MB-435 and 435.neo as reported previously (14), whereas decreasing levels of MMPs were observed in the conditioned medium of all 435 cells treated with flavopiridol (Fig. 4B). These data suggested that increased c-erbB-2 expression in 435.eB cells can lead to increased secretion of MMPs as reported earlier (14) and that flavopiridol can inhibit the expression of c-erbB-2 and, therefore, decrease the secretion of MMPs, which may be important for the decrease in invasion and metastatic ability of breast cancer cells exposed to flavopiridol. However, the decline in MMPs 2 and 9 could be a direct effect on MMPs and not necessarily dependent on the down-regulation of c-erbB-2, which requires further investigation. Whether flavopiridol could have any effect on their ability to invade through matrigel was subsequently investigated as described below.

Inhibition of Cell Invasion by Flavopiridol. It has been reported earlier that c-erbB-2 transfected MDA-MB-435 (435.eB1 and 435.eB4) cells secret MMPs, and these cells acquire invasion and metastatic potential compared to parental cells (435) or cells transfected with control plasmid (435.neo; Ref. 14). We have performed cell invasion assays using these breast cancer cells treated with flavopiridol, which showed inhibition of cell invasion through matrigel within 18 h (Fig. 6), suggesting that the down-regulation of c-erbB-2 followed by the down-regulation of MMPs may play a direct role in the invasion characteristic of these cells when exposed to flavopiridol. The percent inhibition in cell invasion was found to be higher in parental cells, which secret less MMPs compared to 435.eB1, which secrets higher amounts of MMPs and, thus, the residual amount of MMPs observed in flavopiridol treated 435.eB1 remains relatively higher compared to parental cells, which could explain the differential effect on cellular invasion among these cell lines. Collectively, these results provide strong evidence for the antitumor as well as anti-invasive properties of breast cancer cells when exposed to flavopiridol irrespective of c-erbB-2 overexpression, suggesting broader application of flavopiridol in breast cancer.
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DISCUSSION

Flavopiridol is known to potently inhibit the activity of multiple cyclin-dependent kinases. It has been reported to inhibit cell growth of several cancer cell lines (1, 5, 20, 21). A previous report showed that whereas flavopiridol caused cell cycle arrest in G1 and G2 in PC3 cells, apoptosis of SUDHL4 cells occurred without evidence of cell cycle arrest, suggesting that antiproliferative activity of flavopiridol may be different in different cell types from cell cycle arrest to apoptosis (22). In our study, we found that flavopiridol can inhibit the growth of MDA-MB-435, 435.neo, and 435.eB transfectants. To explore if the inhibition of cell growth observed in flavopiridol-treated cells may be due to apoptotic cell death, we used several methods to detect apoptosis in our system. The nucleosomal DNA ladder has been widely used as biochemical markers of apoptosis for several years (23). In addition, the cleavage of Caspase-3 (CPP32) and poly (ADP-ribose) polymerase (PARP) has also been used as early markers of apoptosis (24, 25).

Using these various techniques, we found induced apoptosis in the MDA-MB-435, 435.neo and 435.eB transfectants treated with flavopiridol. DNA ladder formation, CPP32 activation, and PARP cleavage were observed in the breast cancer cells after treatment with flavopiridol for 24 h. Our results clearly suggest that flavopiridol can inhibit the growth of breast cancer cells and induce apoptosis. These results are also consistent with other reports documenting that apoptosis was observed in flavopiridol-treated head and neck cancer cells, lymphoid cells, umbilical vein endothelial cells, leukemia cells, and lung cancer cells (5, 21, 26–28).

It has been demonstrated that Bcl-2 and Bax play a major role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death. Bcl-2 protects cells from apoptosis, whereas increased expression of Bax can induce apoptosis (9, 10). It has also been found that the ratio of Bax:Bcl-2, rather than Bcl-2 alone, is important for the survival of drug-induced apoptosis in leukemia cell lines (10). In our study, a decrease in Bcl-2 expression and up-regulation of Bax expression were observed in breast cancer cells after treatment with flavopiridol for 24 h, corresponding with the induction of apoptosis after flavopiridol treatment. These changes, however, were not related with the status of c-erbB-2 expression. Our results suggest that up-regulation of Bax and down-regulation of Bcl-2 may be one of the molecular mechanisms through which flavopiridol induces apoptosis. However, further in-depth studies are warranted given the importance and potent biological activity of flavopiridol. It is important to note that Konig et al. (21) have found a significant down-regulation of Bcl-2 only after long exposure and a higher concentration of flavopiridol, but two other studies could not find any changes in the levels of Bcl-2 (20, 23). Our studies show some modest down-regulation of Bcl-2, which could either be due to the differences in the cell lines used or this modest effect could be due to the concentration of flavopiridol and the duration of treatment. Further studies with higher concentrations and longer exposure time may provide results similar to the findings obtained by Konig et al. (21). The modulation of cdk as observed previously (1, 3, 26) and its association with the induction of apoptosis is still unknown, but the apparent modest down-regulation of Bcl-2 and up-regulation of Bax may be important for the induction of apoptosis elicited by flavopiridol.

Overexpression of c-erbB-2 has been found in breast cancers, and it has been correlated with lymph node metastasis in cancer patients (29, 30). Tan et al. (14) introduced the human c-erbB-2 gene into the very low c-erbB-2-expressing MDA-MB-435 human breast cancer cells and established 435.eB transfectants that express high levels of c-erbB-2. They compared the metastatic phenotypes of parental MDA-MB-435 cells and the 435.eB transfectants and found that overexpression of c-erbB-2 can enhance the MMP secretion and metastatic potential of MDA-MB-435 human breast cancer cells. In our study, we found that flavopiridol significantly inhibited c-erbB-2 expression (55% to 90%) in the 435.eB transfectants and parental MDA-MB-435 cells, including 435.neo. Because MMPs were previously found to be up-regulated by c-erbB-2 transfection and overexpression, we also compared the secretion of MMPs in 435.eB transfectants and MDA-MB-435 with and without flavopiridol treatment. Higher levels of MMPs in 435.eB transfectants than in parental MDA-MB-435 were observed, corresponding with a higher metastatic potential of 435.eB transfectants as previously reported (14). After treatment with flavopiridol, the levels of MMPs decreased in 435.eB transfectants and MDA-MB-435 parental cells. The decrease in MMPs may be responsible for the inhibition of cell invasion observed in this system (Fig. 6). It is important to note that we were able to observe inhibition of cell invasion in all cell lines within 18 h in our assay system, suggesting that the inhibition of cell invasion may be directly responsible for the biological effect of flavopiridol. The inhibition of cell invasion appears not to be due to cell growth inhibition or apoptosis as suggested by the duration of the experiment and also based on parallel experiments where the initial plating cell density was compensated for these variables. Our results suggest that flavopiridol can inhibit the expression of c-erbB-2 and, in turn, decrease the secretion of MMPs in breast cancer cells, which, in turn, inhibits cell invasion and further suggests that flavopiridol may also inhibit metastatic properties of breast cancer cells. However, the down-regulation of MMPs could be independent of c-erbB-2 in flavopiridol-treated breast cancer cells; this requires further in-depth investigation.

Fig. 6 Matrigel cell invasion assay showing inhibition of cell invasion through matrigel induced by flavopiridol, where the data are presented as percent inhibition of cell invasion relative to control untreated cells.
In conclusion, our results demonstrated that flavopiridol inhibited the growth of breast cancer cells, regulated the expression of apoptosis-related genes, and induced apoptosis in MDA-MB-435 breast cancer cells. Furthermore, flavopiridol inhibited the expression of c-erbB-2, the secretion of MMPs, and cell invasion, suggesting that flavopiridol may also inhibit metastasis of breast cancer. Flavopiridol, thus, may be an effective chemotherapeutic or preventive agent against breast cancer. However, much remains to be studied about the molecular mechanisms of flavopiridol as an anticancer agent in vivo.

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