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Development of drug resistance is reduced with idarubicin relative to other anthracyclines

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Multidrug resistance (MDR) is associated with poor prognosis in leukemia, and anthracyclines, which are used in the treatment of leukemia, are associated with the expression of P-glycoprotein and the development of MDR. We report here that idarubicin, a new anthracycline approved for use in the treatment of acute myelogenous leukemia (AML), did not induce P-glycoprotein expression in the K562 human leukemia cell line under conditions where daunorubicin, doxorubicin and epirubicin did induce expression of P-glycoprotein. The P-glycoprotein expressing, multidrug resistant sublines developed to daunorubicin (K/DNR), doxorubicin (K/DOX) and epirubicin (K/EPR) were cross-resistant to the other anthracyclines and to vinblastine, taxol, colchicine and actinomycin D, but were not resistant to idarubicin or etoposide. The idarubicin treated subline, K/IDA, was only resistant to taxol but was 12-fold sensitized to etoposide, suggesting that idarubicin had affected topoisomerase II in this subline.

Key words: AML, etoposide sensitization, *in vitro*, idarubicin, multidrug resistance, P-glycoprotein.

Introduction

While anthracyclines are among the most widely used and most active chemotherapeutic agents available for the treatment of acute leukemia, they are often associated with treatment failure due to the development of drug resistance which remains a significant problem. Clinical resistance may occur at initial presentation or at relapse. Thus while many patients will achieve complete remission, many will relapse with resistant disease. Development of clinical resistance and poor prognosis has been correlated with the expression of the *mdr-1* gene or its product P-glycoprotein.[1–4] Further, expression of P-glycoprotein has often been reported to be increased at relapse compared to initial presentation.[1,5] *In vitro* studies demonstrate that P-glycoprotein, a 170 kDa membrane protein, acts as a drug efflux pump and confers resistance to a wide range of natural product drugs.[6]

A number of recently published clinical studies suggest that idarubicin is superior to the parent anthracycline, daunorubicin, for the treatment of acute myelogenous leukemia (AML).[7] While the mechanism of action of idarubicin does not appear to be different from other anthracyclines,[8] the deletion of the methoxy group at position 4 makes idarubicin more lipophilic.[9] This results in a more rapid cellular uptake and higher internal drug concentration than daunorubicin.[10] It is suggested that this increased drug accumulation is the main reason for the increased cytotoxicity demonstrated by idarubicin. Further, idarubicin has been shown to have activity in relapsed or refractory leukemia and significantly higher rates of complete remission and longer survival have been shown for patients treated with idarubicin/cytarabine compared with daunorubicin/cytarabine.[11] Idarubicin treatment has even been found to reduce the number of bone marrow cells expressing P-glycoprotein.[12] *In vitro* studies suggest that the improved response to idaorubicin may be due to its ability to circumvent the activity of P-glycoprotein because of its increased lipophilicity.[8,13] However, little information is available on whether idarubicin, like the other anthracyclines, promotes the development of P-glycoprotein mediated multidrug resistance (MDR).

Previously we have reported the development of drug resistance and expression of P-glycoprotein in the K562 human leukemia cell line in response to treatment of with low clinically relevant levels of the anthracycline epirubicin.[14] The K562 cell line is ideal for these studies since it is a pluripotent stem cell line developed from a patient with acute myelogenous leukemia[15] and retains the ability to differentiate in response to many agents.[16] We have therefore used this cell line to compare the four

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anthracyclines, idarubicin, daunorubicin, doxorubicin and epirubicin, for their potential to cause the development of drug resistance.

Materials and methods

Cell lines

The K562 human myeloleukemia cell line[15] was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium (Cytosystems, Sydney, Australia) supplemented with 10% fetal calf serum (Cytosystems), 20 mM HEPES (Cytosystems) and $NaHCO_3$ (0.85 g/l) at 37°C in a humidified atmosphere with 5% CO_2. Exponentially growing cells were used for all experiments.

Resistant sublines were derived by intermittently exposing the parental K562 cells to 20 ng/ml of either idarubicin, daunorubicin, doxorubicin or epirubicin for 3 days over a 2 month period. This produced four sublines K/IDA (idarubicin), K/DNR (daunorubicin), K/DOX (doxorubicin) and K/EPR (epirubicin) depending on the drug used in treatment. Sublines were maintained by treatment for 3 days with the same drug every 6 weeks. All cultures were free of mycoplasma.

Cytotoxicity assays

Sensitivity to drugs was determined using the MTT cell viability assay as previously described.[17] Briefly, cells (3×10^4) were plated in triplicate in 200 µl media containing 2-fold serial dilutions of drug. After 4 days incubation, cell viability was determined using MTT. The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration which resulted in a 50% reduction in cell viability. Relative resistance was calculated by dividing the IC_{50} obtained for the resistant subline by the IC_{50} obtained for the K562 parental cell line. Reversal of resistance was determined by incubating cells in the presence and absence of verapamil (10 µM) in a cytotoxicity assay. The IC_{50} was determined and fold reversal was calculated by division of the IC_{50} without reversing agent by the IC_{50} in the presence of reversing agent. Determinations were performed in triplicate and all assays were repeated at least twice. Statistical significance was determined by Student's *t*-test.

P-glycoprotein expression

Flow cytometry. Cells (10^5) were incubated in duplicate with 2 µg MRK16 monoclonal antibody (kindly donated by Dr T Tsuruo) or control mouse IgG2a monoclonal antibody and assayed by flow cytometry as previously described.[14]

Western blots. Membrane fractions were prepared, subjected to electrophoresis and probed with C219 monoclonal antibody (Centocore, Malvern, PA) as previously described.[17] Five micrograms of protein was applied for each sample and equal protein loading was confirmed by Coomassie staining of duplicate gels.

Drugs and reagents

Idarubicin and epirubicin were from Farmitalia (Melbourne, Australia), daunorubicin, vinblastine and cisplatin were from David Bull (Melbourne, Australia), doxorubicin was from Delta West (Bentley, Australia), etoposide was from Bristol (Sydney, Australia), 3,4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), verapamil, taxol, colchicine and actinomycin D were purchased from Sigma (St Louis, MO). All other chemicals were AR grade.

Results

Development of drug resistance

K562 cells were treated for 3 days with 20 ng/ml of idarubicin (36 nM), daunorubicin (35 nM), doxorubicin (34 nM) or epirubicin (34 nM) over a period of 2 months in an attempt to mimic clinical conditions. This drug concentration was well below the IC_{50} values obtained for these drugs in the 4 day MTT cytotoxicity assay which gave an IC_{50} of idarubicin 117 ± 41 nM ($n = 6$), daunorubicin 220 ± 69 nM ($n = 7$), doxorubicin 600 ± 160 nM ($n = 4$) and epirubicin 397 ± 92 nM ($n = 4$). Treatment resulted in the development of four sublines K/IDA, K/DNR, K/DOX and K/EPR corresponding to the drug used (idarubicin, daunorubicin, doxorubicin and epirubicin, respectively). After six treatments cell growth over 3 days in the absence of drug was similar to cell growth of the parental K562 cells (Figure 1). However, in the presence of the selecting drug cell growth of the K/IDA subline was completely inhibited, while the K/DNR subline was substantially inhibited (60%). Cell growth of the K/DOX and K/EPR were only slightly reduced (30%).

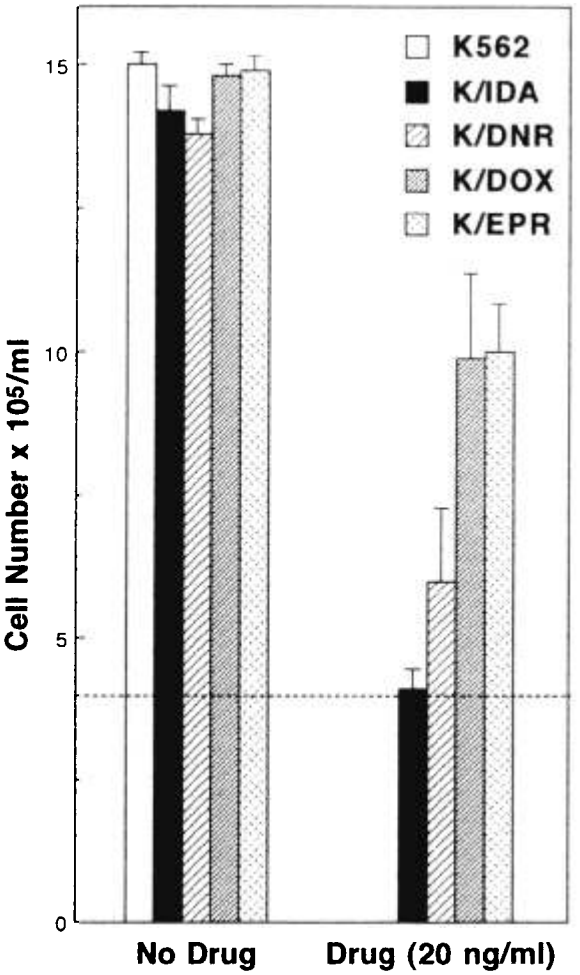

Figure 1. Subline cell growth rate in the presence or absence of drug. K562 cells (open bar) and anthracycline treated sublines (K/IDA, K/DNR, K/DOX, K/EPR) were seeded at 4×10^5 cells/ml (dotted line) in RPMI or RPMI containing 20 ng/ml of the selecting drug. Cells were counted after 3 days incubation. Results are the mean of duplicate counts.

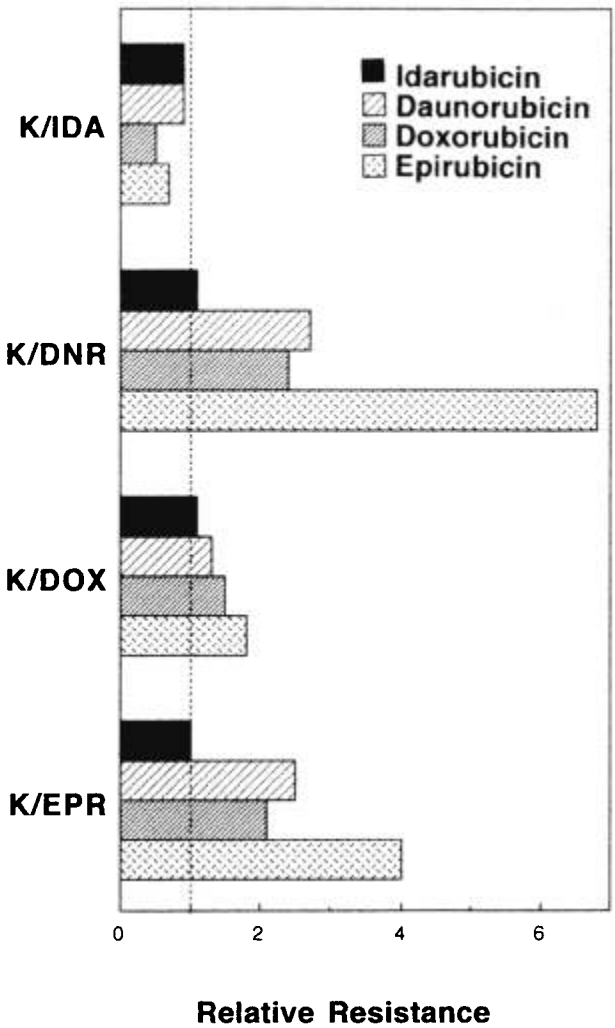

Figure 2. Relative resistance of K562 sublines to anthracyclines. Cytotoxicity assays were performed on K562 cells and sublines and the IC_{50} determined. Relative resistance = IC_{50} of the subline/IC_{50} of parental K562 cells. Results are the mean of at least two experiments performed in triplicate. Dotted line indicates the relative resistance of 1 for the K562 cells.

Resistance to anthracyclines

Sublines were tested for resistance to the selecting drug and to other anthracyclines (Figure 2). None of the sublines showed resistance to idarubicin. Further, the K/IDA subline showed no resistance to any of the anthracyclines. The K/DNR and K/EPR sublines showed most resistance and this was greater to daunorubicin and epirubicin (2.5- to 6.8-fold; $p \leq 0.02$; $n \geq 2$). However, the K/DOX subline showed only low levels of cross-resistance to epirubicin (1.7-fold; $p < 0.01$; $n = 5$), daunorubicin (1.5-fold; not significant; $n = 2$) or doxorubicin (1.5-fold; not significant; $n = 2$).

Cross-resistance

Figure 3 summarizes the cross-resistance of the four sublines to drugs in the MDR family and shows that the K/DNR subline was the most resistant subline followed by the K/EPR subline. The K/DNR subline was cross-resistant to vinblastine (11.8-fold), taxol (25.0-fold), actinomycin D (15.7-fold) and colchicine (3.7-fold). The K/EPR subline showed a similar cross-resistance pattern. The K/DOX subline was less resistant to these drugs and the K/IDA subline was resistant only to taxol (2-fold; $p < 0.05$; $n = 2$), and tended to be resistant to actinomycin D (2-fold; not significant; $n = 2$). Further, the K/IDA subline

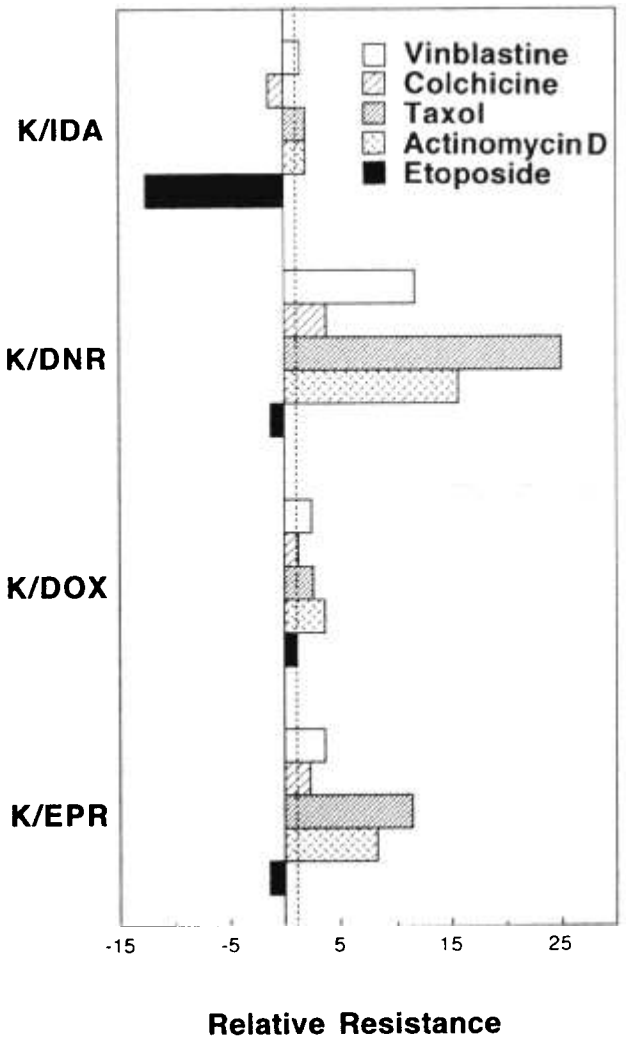

Figure 3. Cross-resistance of K562 sublines to MDR drugs. Conditions were those described in the legend to Figure 2.

was approximately 12- fold sensitized to etoposide ($p < 0.01$, $n = 2$). The sublines were not cross-resistant to the non-MDR drug cisplatin (results not shown).

Expression of P-glycoprotein

Flow cytometry, using MRK16 monoclonal antibody specific for an external epitope on P-glycoprotein,[18] showed that the K DNR and K EPR sublines expressed high levels of P-glycoprotein while the K DOX expressed a lower level (Figure 4). P-glycoprotein was not detected in the K IDA subline. These results were confirmed using C219 monoclonal antibody, specific for an internal cytoplasmic site on P-glycoprotein,[19] and Western blot analysis (results not shown).

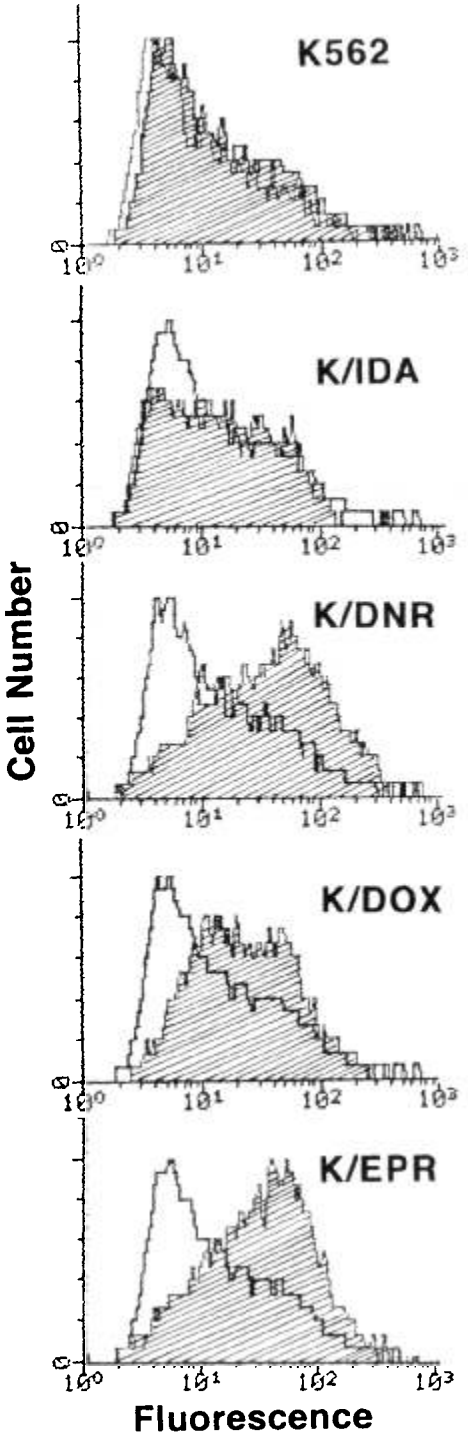

Figure 4. Flow cytometry analysis of P-glycoprotein expression with MRK16 monoclonal antibody. K562 cells (hatched profile) were incubated with MRK16 monoclonal antibody and fluorescence compared to the negative control antibody (- - - -). Each drug resistant subline incubated with MRK16 (hatched profile) was compared to the parental K562 cells (- - - -). All sublines gave the same negative control antibody profile. Fluorescence profiles are representative of two different experiments performed in duplicate.

As idarubicin was more cytotoxic than the other anthracyclines, K562 cells were also treated with lower concentrations of drug, 5 ng/ml (9 nM) and 10 ng/ml (18 nM). The sublines developed were similar to the K/IDA, and did not express P-glycoprotein (results now shown).

Effect of verapamil

The effect of verapamil, an inhibitor of P-glycoprotein mediated drug efflux, was examined. Figure 5 shows that while verapamil had little effect on the K562 cell line, verapamil sensitized the drug resistant sublines to the effects of taxol. Even the minimal resistance to this drug demonstrated by the K/IDA and the K/DOX sublines was sensitized by co-incubation with 10 μM verapamil.

Discussion

While the clinical success of idarubicin may be due to its increased lipophilicity, increased intracellular drug concentration and the circumvention of P-glycoprotein mediated efflux,[13] the results presented here suggest that idarubicin offers the further advantage of not inducing P-glycoprotein expression as readily as the other anthracyclines. After six treatments with low levels of the four anthracyclines, the relative expression of P-glycoprotein in the sublines was K/DNR ≥ K/EPI > K/DOX > K/IDA. This expression does not simply correspond to the relative cytotoxicity of these drugs to the K562 cells which is idarubicin > daunorubicin > epirubicin > doxoru-bicin, similar to that reported in other cell lines.[20] The increased cytotoxicity of idarubicin alone does not account for the lack of induction of P-glycoprotein since treatment of K562 cells with lower doses of idarubicin (5 or 10 ng/ml) did not induce expression of P-glycoprotein.

The MDR induced in the K/DNR, K/EPR and K/DOX sublines was typical of that associated with P-glycoprotein expression with cross-resistance to anthracyclines (Figure 2), *Vinca* alkaloids and taxol (Figure 3) and sensitization by verapamil (Figure 5). These sublines were not cross-resistant to etoposide, a drug which is often associated with P-glycoprotein expression (Figure 3). This may be because etoposide is a poor substrate for P-glycoprotein[21] and these sublines have a relatively low level of resistance. The lack of etoposide resistance also indicates that MRP (multidrug resistance-associated protein), the recently characterized multidrug transporter, is not involved since MRP confers a significant level of resistance to etoposide.[22,23] Although the high level of resistance to taxol (Figure 3) was unexpected, this is consistent with the greater success of taxol in the treatment of solid tumors than in the treatment of leukemia.

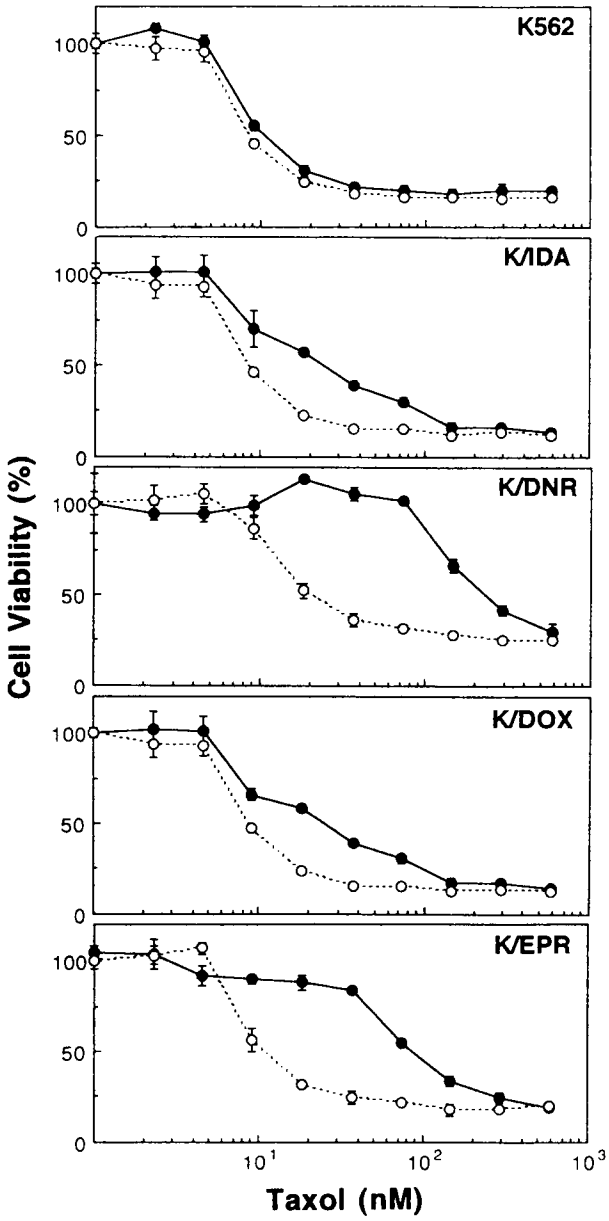

Figure 5. Effect of verapamil on taxol cytotoxicity. K562 cells or the drug resistant sublines were assayed for resistance to taxol in the absence (closed symbols) or presence (open symbols) of 10 μM verapamil. Points are the means of triplicate determinations.

The K/IDA subline was 12-fold more sensitive to etoposide than the K562 parental cell line, suggesting that it may have altered topoisomerase II since etoposide is a topoisomerase II poison. Whether this alteration is also associated with the 2-fold re-

sistance to taxol observed in this subline remains to be determined. However, there is a possible link between the cytotoxic action of taxol[24] and etoposide,[25] since both cause cell killing by gene regulated apoptosis. In light of the increased sensitivity to etoposide in the K/IDA subline, it is of interest that idarubicin in combination with cytarabine and etoposide has proved successful in the treatment of refractory of relapsed AML patients.[11,26]

Conclusion

In addition to the known advantages of idarubicin, such as its increased cytotoxicity both in MDR and non-MDR expressing cells, idarubicin is also less likely to cause the development of multidrug resistance, especially in comparison to daunorubicin and epirubicin. The resistant subline resulting from idarubicin treatment showed only low level resistance to taxol and it was 12-fold sensitized to etoposide. Therefore etoposide may be more effective as part of second line therapy following initial treatment with a combination containing idarubicin.

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