

# Combined Effects of Streptozotocin and Mitozolomide against Four Human Cell Lines of the Mer<sup>+</sup> Phenotype

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## ABSTRACT

The majority of human tumor cell lines are proficient in the repair of guanine O<sup>6</sup>-alkylations (designated Mer<sup>+</sup>) and are thus capable of preventing the cytotoxic effects of chloroethylating agents. It has been proposed that in these cells guanine O<sup>6</sup>-chloroethylations are rapidly removed by the enzyme O<sup>6</sup>-alkylguanine DNA alkyltransferase before the formation of DNA interstrand cross-links can occur. In this study pretreatment of four Mer<sup>+</sup> human cells (A2182 lung carcinoma, A375 melanoma, HT-29 colon carcinoma, and IMR-90 normal lung fibroblasts) with the DNA methylating agent streptozotocin apparently saturates the monoadduct repair system and allows mitozolomide to form interstrand cross-links in these cells. The inhibition of the alkyltransferase results in the continued presence of guanine O<sup>6</sup>-chloroethylations which then undergo a series of reactions that lead to DNA interstrand cross-link formation. As observed by colony forming assays, streptozotocin pretreatment causes a dramatic increase in the sensitivity of these four Mer<sup>+</sup> cell lines to the cytotoxic effects of mitozolomide. These results indicate that a combination of streptozotocin pretreatment followed by mitozolomide may be useful in the treatment of human cancer.

## INTRODUCTION

Chloroethylating agents such as the chloroethylnitrosoureas are thought to exert their antitumor activity by an initial chloroethylation at position O<sup>6</sup> of guanine which is followed by a series of reactions that leads to a DNA interstrand cross-link with a cytosine on the opposite strand (1). Cells that are proficient in the repair of guanine O<sup>6</sup>-alkylations (Mer<sup>+</sup>) do not allow DNA interstrand cross-links to form and are resistant to the cytotoxic effects of CLETNU<sup>2</sup> (2). In contrast, cells that are deficient in the repair of guanine O<sup>6</sup>-alkylation (Mer<sup>-</sup>) allow DNA interstrand cross-links to form and are sensitive to the cytotoxic effects of CLETNU (2, 3). We have previously shown that Mer<sup>-</sup> cells are also sensitive to the effects of 8-carbamoyl-3-(2-chloroethyl)-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (mitozolomide), whereas Mer<sup>+</sup> cells are resistant (4, 5), and this again correlates with the presence of DNA interstrand cross-links in Mer<sup>-</sup> cells and their absence in Mer<sup>+</sup> cells (4, 5). Hence, although the route of production of chloroethylating species is different for each of these agents their mechanism of action is thought to be similar (4, 6, 7). Mitozolomide, however, has been shown to produce a 4-fold higher peak plasma level than the CLETNU (in terms of area under the curve) in both mice and humans (8). Furthermore, mitozolomide was found to have marked antitumor activity against human melanomas, sarcomas, and lung and colon cancers in both the 6-day subrenal capsule assay in immunocompetent mice and on s.c. growing tumors in athymic nude mice (9). Additionally,

mitozolomide retains antitumor activity when administered by the p.o. route (10). These results suggested that mitozolomide may have distinct advantages over the CLETNU currently used in the clinic.

In contrast to their activity in animal tumors, CLETNU have proved somewhat disappointing in the clinic with a general overall response rate of around 20-30% (11). It appears that a major reason for this failure may be due to the finding that the majority of human tumor cells, as well as normal human cells, have guanine O<sup>6</sup>-alkylguanine DNA alkyltransferase activity. In fact, 70-80% of all human cells tested for this repair activity are Mer<sup>+</sup> (12). This repair protein is known to react in a stoichiometric manner and, hence, is depletable. With this in mind we have tried to circumvent the natural resistance of such human tumor cells by saturating out this repair activity. We have recently reported that pretreating Mer<sup>+</sup> cells with the methylating agent MNNG sensitizes them to the effects of chloroethylating agents (13) and in particular mitozolomide (5), but not to other alkylating agents that react primarily at position N<sup>7</sup> of guanine (14). In addition, this pretreatment allowed DNA interstrand cross-links to form in Mer<sup>+</sup> cells where previously they had been undetected (5, 13). Because of its high carcinogenic potential, the utilization of MNNG in the clinic is not likely, so we have thus searched for clinically useful alternatives which may sensitize resistant cells to the effect of mitozolomide.

STZ appears an ideal alternative to MNNG; it has antitumor activity in its own right (15), and is capable of methylating position O<sup>6</sup> of guanine in rat tissue DNA (16). Repair of guanine O<sup>6</sup>-methylations is thought to be important in saturating out O<sup>6</sup>-alkylguanine DNA alkyltransferase in human cells (13). In this study we would like to report that pretreatment of Mer<sup>+</sup> cells with the methylating agent STZ sensitizes these cells to the cytotoxic effects of mitozolomide. The possible use of a methylating agent pretreatment followed by a chloroethylating agent in the clinic will be discussed in light of our results.

## MATERIALS AND METHODS

**Cell Culture.** HT-29, a human colon carcinoma cell line, was originally isolated by J. Fogh (17). IMR-90, a normal human fibroblast cell line derived from embryonic lung, was obtained from W. Nichols, Institute of Medical Research, Camden, NJ. A2182, a human lung carcinoma, and A375, a human melanoma, were obtained from R. Day, Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda, MD. All cells were cultured for alkaline elution studies, and colony formation assays, in a similar manner to that described previously (3-5).

For alkaline elution studies the DNA of all cells was labeled by growing the cells in 0.02 μCi [<sup>14</sup>C]thymidine/ml (New England Nuclear; specific activity, 52 mCi/mmol). L1210 mouse leukemia cells, which were used as internal standards in the alkaline elution assays, were grown in suspension culture in RPMI 1630 supplemented with 15% heat inactivated (60°C, 45 min) fetal bovine serum. The DNA of L1210 cells was labeled by growing 3 × 10<sup>5</sup> cells/ml for 20 h in RPMI 1630 supplemented with 0.05 μCi [<sup>3</sup>H]thymidine/ml (New England Nuclear; specific activity, 20 Ci/mmol) and 10<sup>-6</sup> M unlabeled thymidine.

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<sup>2</sup> The abbreviations used are: CLETNU, chloroethylnitrosoureas; mitozolomide, 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one; STZ, streptozotocin; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

**Drug Treatment.** Mitozolomide was a gift of Professor M. F. G. Stevens, Department of Pharmacy, University of Aston, Birmingham, England. Streptozotocin (NSC 805998) was obtained from the Drug Developmental Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. In all cases a 1-h streptozotocin drug exposure and a 2-h mitozolomide drug exposure were used. The pretreatment protocol has previously been described in detail (5, 13).

**Alkaline Elution.** The alkaline elution procedure used in these experiments has been described in detail, and recently reviewed (18). Two modifications of the procedure were used.

In summary, DNA interstrand cross-links were detected by the following procedure. Cells were irradiated at 0°C with 300 rads  $\gamma$ -irradiation from a  $^{137}\text{Cs}$  source (J. L. Shepherd Co., Glendale, CA; dose rate, 314 R/min).  $^3\text{H}$ -L1210 internal standard cells which had received 300 rads on ice were added to the cell suspension. Elution conditions were as follows. Cells were layered onto 0.8  $\mu\text{m}$  pore size polycarbonate filters (Nucleopore, Pleasanton, CA), lysed with 2% sodium lauryl sulfate containing 0.1 M glycine, 0.02 M EDTA, pH 10.0, and digested with 2 ml of 0.5 mg proteinase K/ml in lysis solution (EM Laboratories, Darmstadt, Federal Republic of Germany); and the DNA was eluted from the filters by pumping a 0.02 M EDTA solution, adjusted to pH 12.1 with tetrapropylammonium hydroxide (RSA Corp., Elmsford, NY) and containing 0.1% sodium lauryl sulfate, through the filters at approximately 6 ml/h. Five 1-h fractions were collected and DNA interstrand cross-linking indices were calculated using the equation:

$$\text{Cross-link index} = \sqrt{\frac{1-r_0}{1-r}} - 1$$

where  $r$  is the retention of the DNA that was pretreated with a methylating agent, followed by mitozolomide treatment, and irradiated, and  $r_0$  is the retention of the DNA that was treated with a methylating agent only and irradiated (13, 19). To express cross-linking frequency in rad equivalents, the following equation was used:

$$\text{Cross-link frequency (rad equivalents)} = \sqrt{\frac{1-r_0}{1-r}} - 1 \times 300 \text{ rads}$$

(plus equivalents of methylating agent induced single strand breaks). In this manner, the single strand breaks introduced by the pretreatment dose of methylating agent are being taken into account (19).

**Colony Forming Assays.** IMR-90 cells and A375 were seeded in 25-cm<sup>2</sup> plastic flasks (Corning Glass Works, Corning, NY), and HT-29 and A2182 were seeded in 25-cm<sup>2</sup> plastic flasks (Costar, Cambridge, MA). The details of the colony formation assays have been described previously (3-5).

## RESULTS

In the following experiments, our working hypothesis was that STZ pretreatment of Mer<sup>+</sup> cells would increase the cytotoxicity of mitozolomide by inhibiting a DNA repair process in resistant cells and concurrently allowing mitozolomide to induce DNA interstrand cross-links in these cell lines. We have previously shown that 1 mM STZ only slightly increased the cell kill produced by CLETNU in HT-29 (Mer<sup>+</sup>) cells with no apparent increase in DNA interstrand cross-linking. In this study we have found that the choice of STZ concentration used in pretreating Mer<sup>+</sup> cells is critical for the observed increase in cell kill and detection of DNA interstrand cross-links.

The inhibition of the colony forming ability of four Mer<sup>+</sup> cell lines by STZ is shown in Fig. 1. We have found that each cell line has a different sensitivity to STZ. For example, the concentrations required to produce a 50% inhibition in the colony forming ability of A2182, A375, IMR-90, and HT-29 cells are 1.2, 2.3, 2.6, and 4.2 mM, respectively. Based on these results we have used the following pretreatment concentrations; 1.25 mM for A2182 cells, 2 mM for A375 cells, and 2.5 mM for both IMR-90 and HT-29 cells. The need for the use of different

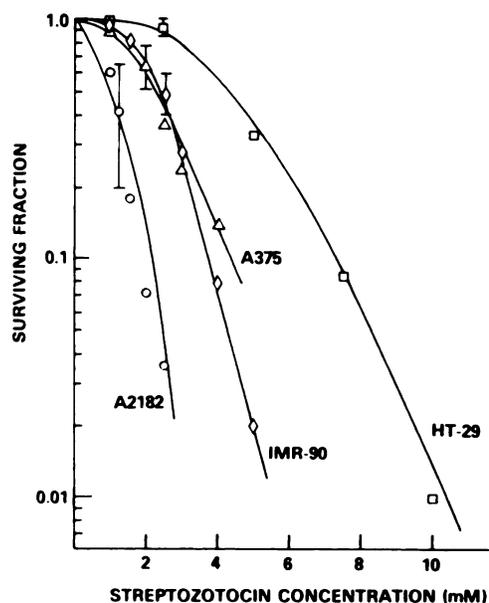


Fig. 1. Inhibition of colony forming ability of A2182, A375, IMR-90, and HT-29 cells by streptozotocin. Points, average of 2 independent experiments of 18 or more replicate plates; bars, SD of the STZ pretreatment concentration of 3 independent experiments.

concentrations of STZ may reflect the different levels of guanine  $O^6$ -alkylguanine DNA alkyltransferase activity that is thought to exist in Mer<sup>+</sup> cell lines (20). Indeed, these results are in agreement with the levels of  $O^6$ -alkylguanine DNA alkyltransferase found in the A2182 and HT-29 cells (21).

Fig. 2 shows the cytotoxicity of mitozolomide to the four human Mer<sup>+</sup> cell lines and compares them with the cytotoxicity of mitozolomide after STZ pretreatment. In all cases there was at least a 1-log synergistic increase in the toxicity of mitozolomide following STZ pretreatment. These survival curves have been normalized for any cytotoxicity produced by STZ alone. To test the ability of STZ to inhibit the  $O^6$ -alkylguanine DNA alkyltransferase repair system, the following experiments were carried out: all four cell lines were treated for 1 h with the indicated concentration of STZ, drug was then removed, and each cell line was exposed to mitozolomide for 2 h. Cells were then incubated in drug-free medium for 6 h to allow formation of mitozolomide induced cross-links. The formation of DNA interstrand cross-links directly reflects the continued presence of guanine  $O^6$ -chloroethylations due to the inhibition of the repair activity. As can be seen from Fig. 3, STZ pretreatment allowed mitozolomide to induce DNA interstrand cross-links in each cell line where previously none had been observed. These data can be seen more clearly in Table 1, which shows the cross-link index in rad equivalents induced by mitozolomide in each of the four cell lines, either with or without STZ.

## DISCUSSION

Mitozolomide is a new chloroethylating agent which is currently undergoing clinical trials in Europe. Its mechanism of action appears to involve chloroethylation of DNA, since cell lines which are deficient in the repair of guanine  $O^6$ -alkylations are sensitive to mitozolomide, whereas cell lines which are proficient in the repair are resistant (4, 5). Mitozolomide and CLETNU thus appear similar in that they require a chloroethylation at position  $O^6$  of guanine to elicit their cytotoxic effect (22). The presence of a  $O^6$ -alkylguanine DNA alkyltransferase repair activity in human tumor cells may make these cells resistant to the effects of chloroethylating agents (2, 3). Rufus

COMBINED EFFECTS OF STREPTOZOTOCIN AND MITOZOLIMIDE

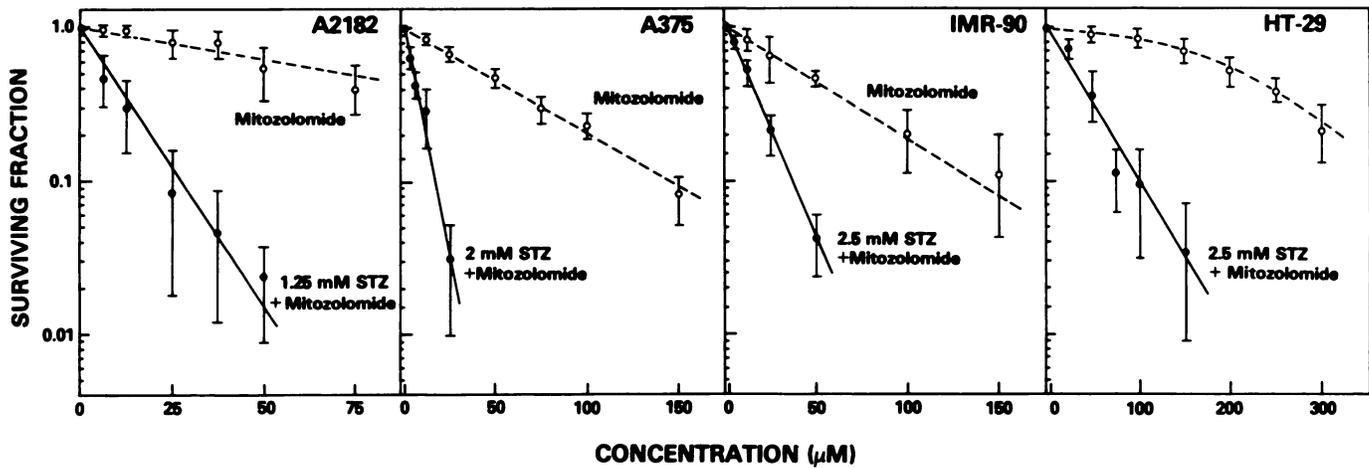


Fig. 2. Inhibition of colony forming ability of each cell line exposed to various concentrations of mitozolomide (O—O) for 2 h at 37°C. Pretreatment of each cell line with indicated concentration of STZ for 1 h followed by mitozolomide (●—●) for 2 h at 37°C. The data of STZ plus mitozolomide have been normalized against the survival of cells exposed to STZ alone. Points, mean of 3 independent experiments and of at least 27 or more replicate plates; bars, SD.

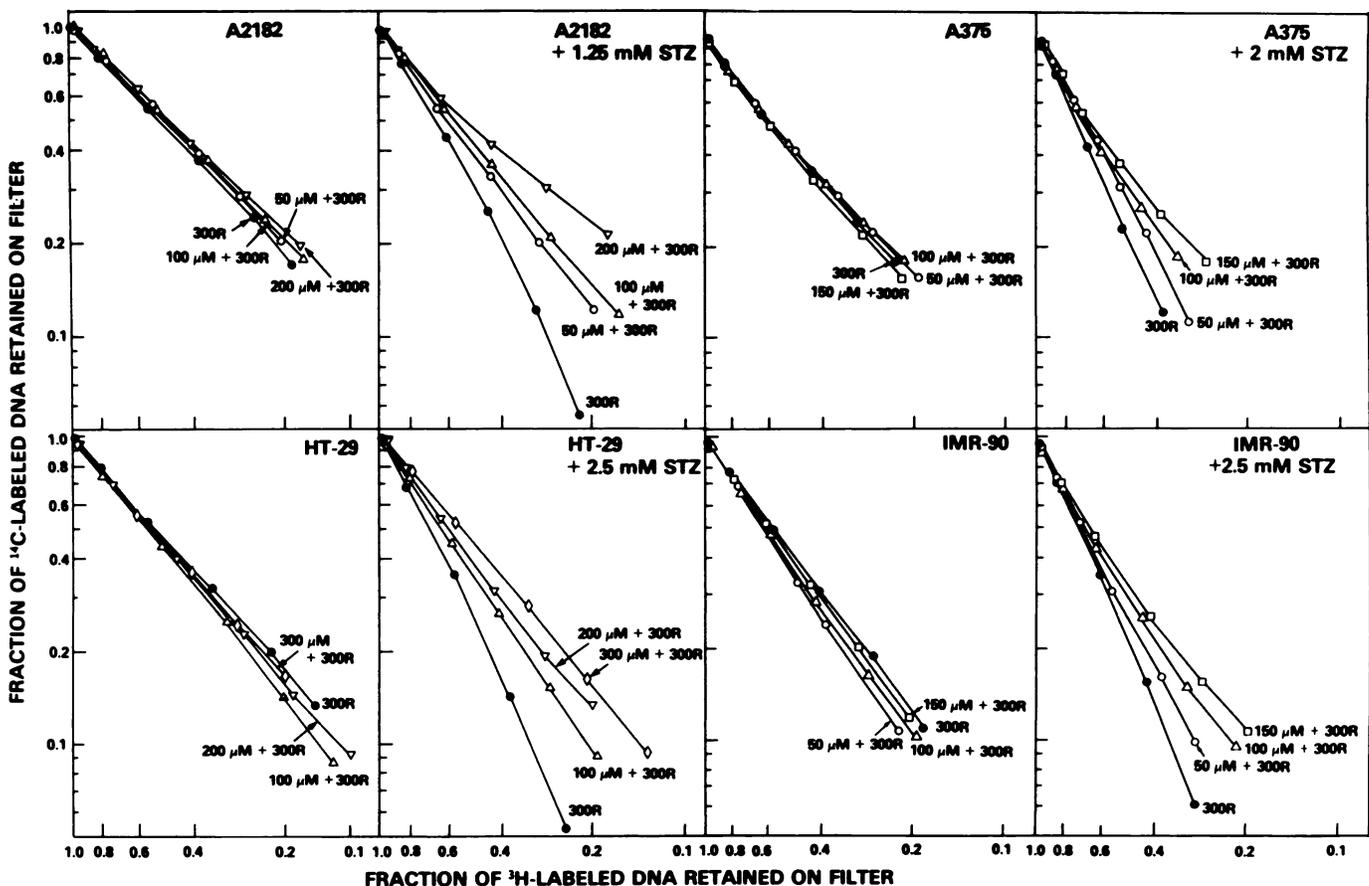


Fig. 3. Alkaline elution assays to test for the formation of DNA interstrand cross-links after mitozolomide treatment with or without STZ pretreatment. Panels and concentrations are as indicated. These profiles are taken from one experiment and are representative of at least three independent experiments.

Day and colleagues at the National Cancer Institute have now tested approximately 90 human cell lines and found that 70–80% of all cell lines tested are of the Mer<sup>+</sup> phenotype.<sup>3</sup> Furthermore, Myrnes *et al.* (23) have recently shown that only 5 of 24 tumors isolated have low O<sup>6</sup>-alkylguanine DNA alkyltransferase levels. From these data, it would be expected that in the clinical situation 70–80% of all human tumors would be resistant to the effects of chloroethylating agents such as the CLETNU and mitozolomide. Consistent with this hypothesis

is the fact that the CLETNU have a general response rate of around 20–30% (11). Recent work has shown that pretreatment of the resistant Mer<sup>+</sup> cells with methylating agents can sensitize these cells to the effects of chloroethylating agents (5, 13). In this study we have reported that pretreatment of Mer<sup>+</sup> cells with STZ, an agent that can methylate position O<sup>6</sup> of guanine (16), thereby depleting the O<sup>6</sup>-alkylguanine DNA alkyltransferase repair activity, can be used to sensitize tumor cells to mitozolomide. This confirms previous work in which Mer<sup>+</sup> cells were sensitized to the effects of mitozolomide (5) and CLETNU (13, 19) by pretreatment with MNNG.

<sup>3</sup> R. Day, personal communication.

Table 1 Interstrand cross-link index (rad equivalents) of four Mer<sup>+</sup> cell lines treated with mitozolomide, with or without STZ pretreatment

Cell line	Concentration (μM)	No STZ pretreatment	STZ pretreatment
A2182	50	1.65 ± 2.14 <sup>a</sup>	14.25 ± 1.65
	100	4.12 ± 2.37	23.50 ± 1.65
	200	6.60 ± 2.31	39.10 ± 6.97
A375	50	1.40 ± 1.21	12.10 ± 2.40
	100	1.40 ± 1.26	20.30 ± 6.00
	150	-1.40 ± 1.20	29.30 ± 5.80
IMR-90	50	-1.99 ± 3.45	8.61 ± 5.10
	100	-1.34 ± 2.32	16.42 ± 11.60
	150	-1.84 ± 1.76	24.02 ± 13.5
HT-29	100	3.90 ± 7.80	13.30 ± 7.50
	200	-2.70 ± 5.00	21.00 ± 7.36
	300	-2.20 ± 3.10	34.40 ± 8.50

<sup>a</sup> Mean ± SD of at least three independent experiments.

The high carcinogenic potential of MNNG exposure made this protocol unsuitable for clinical use. STZ, however, would appear more suitable as a methylating agent with potential clinical use. STZ has been shown to sensitize human tumor cell lines of the Mer<sup>+</sup> phenotype to *N,N*-bis(2-chloroethyl)-*N*-nitrosourea, chloroethylnitrosourea, and chlorozotocin (24). In addition, another distinct advantage of STZ is that it has antitumor activity in its own right and that its toxicology has already been studied in the clinic in detail (15). The work presented here confirms that *in vitro* STZ can sensitize human tumor cells to the cytotoxic effects of mitozolomide to which they had previously been resistant. One problem associated with a methylating agent pretreatment followed by a chloroethylating agent is that you may increase nonselective toxicities such as myelosuppression (11, 15).

There are two possible mechanisms by which STZ may inactivate the repair of *O*<sup>6</sup>-alkylguanine lesions: (a) STZ may directly react with the repair protein itself, thus inactivating it directly, or (b) STZ may act by alkylating the DNA at position *O*<sup>6</sup> of guanine and then the protein which reacts with the alkylated guanine in a stoichiometric fashion is simply depleted. In addition, a combination of the two pathways is possible.

Pretreatment with STZ, which increased the cytotoxicity of mitozolomide, also led to the formation of DNA interstrand cross-links in the four Mer<sup>+</sup> cell lines where previously none had been detected. It is worth noting that the lack of removal of *O*<sup>6</sup>-chloroethyl monoadducts after STZ pretreatment is the reason for the formation of DNA interstrand cross-links, and thus it may be inferred that either the guanine *O*<sup>6</sup>-chloroethylation or the result of DNA interstrand cross-link be responsible for the increase in cytotoxicity.

In summary it would appear that the combination of STZ with the chloroethylating agent mitozolomide is worthy of consideration for use in the clinical situation.

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