Enhancement of Antibody-directed Enzyme Prodrug Therapy in Colorectal Xenografts by an Antivascular Agent


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ABSTRACT

The irregular nature of solid tumor vasculature produces a heterogeneous distribution of antibody-targeted therapies within the tumor mass, which frequently results in reduced therapeutic efficacy. We have, therefore, combined two complementary therapies: Antibody-directed Enzyme Prodrug Therapy (ADEPT), which targets tumor cells, and an agent that selectively destroys tumor vasculature. A single i.p. dose (27.5 mg/kg) of the drug 5,6-dimethylxanthene-4-acetic acid (DMXAA), given to nude mice bearing the LS174T colorectal xenograft, destroyed all but a peripheral rim of tumor cells, without enhancing survival. The ADEPT system, in which a pretargeted enzyme activates a prodrug, consisted of the F(ab)’ fragment of anti-carcinoembryonic antigen antibody ASB7 conjugated to the bacterial enzyme carboxypeptidase G2 and the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid, which was given i.p. in three doses of 500 mg/kg at 72, 84, and 96 h post-conjugate administration (25 units of carboxypeptidase G2). The anti-enzyme conjugate could be selectively retained at approximately twice the control levels by administration of the antivascular agent at the time of optimal conjugate localization within the tumor (20 h post-conjugate administration), as demonstrated by gamma counting, phosphor plate image analysis, and active enzyme measurement. This resulted in significantly enhanced tumor growth inhibition in groups of six mice, compared to conventional ADEPT therapy, with no concomitant increase in systemic toxicity. In a separate experiment, aimed at trapping the drug 5,6-dimethylxanthene-4-acetic acid; CMDA, 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid.

INTRODUCTION

The heterogeneous structure and physiology of solid tumors creates a major delivery problem for many systemically administered anticancer agents. As the tumor grows, the vasculature becomes leaky, with shunts and sluggish blood flow. This produces increased pressure and low pO2 within all but the periphery of the tumor in both animal models and man, reducing the potential efficacy of many cancer treatments (1). Antibody-targeted therapies have significantly improved selective delivery of anticancer agents (2), but this advantage is counteracted by both their slow diffusion rate through the tumor compared to smaller drug molecules and their heterogeneous distribution pattern, created by the tumor blood supply (3).

ADEPT3 is a two-phase system that was developed in an attempt to overcome some of these delivery problems (4). In this approach, a nontoxic exogenous enzyme is conjugated to an antitumor antibody, administered systemically, and allowed to localize within the tumor and clear from normal tissues. A relatively nontoxic prodrug is then given, which is selectively converted to a toxic drug at the tumor site by the pretargeted enzyme. There are several advantages to this system: the active drug is a small molecule that can readily diffuse through the tumor to reach both antigen-positive and -negative cells, one enzyme molecule can activate many prodrug molecules, and the generation of the active drug within the tumor reduces systemic toxicity. Many different ADEPT systems have been developed (5, 6). One such system uses the bacterial enzyme CPG2, which has no mammalian homologue. The enzyme was conjugated to a F(ab)’2 fragment of the anti-CEA antibody ASB7 and used in combination with various mustard prodrugs (7, 8). This has produced inhibition of tumor growth in a range of xenograft models, including choriocarcinoma and colon and breast carcinomas (6), and given partial remissions in a Phase I clinical trial (9, 10). However, even with the advent of prodrugs that release more potent drugs, it will probably be necessary to give repeated ADEPT doses for effective therapy, increasing the problem of both antibody and enzyme immunogenicity. Repeat doses have been administered in patients, but this required concomitant treatment with the immunosuppressive drug cyclosporin (9). It would, therefore, be advantageous to combine ADEPT with a complementary or synergistic therapy that would selectively destroy those tumor cells that are inaccessible or more resistant to antibody-targeted treatments. A further therapeutic gain might be achieved if increased amounts of either conjugate or prodrug could be retained selectively at the tumor site without increasing systemic toxicity.

Specific destruction of tumor vasculature has greater therapeutic potential than attacking the tumor cells directly, but current strategies frequently fail to destroy all functional vessels, especially those around the periphery of the tumor (11). This viable rim, from which the tumor continues to grow, has proved refractory to most treatments used thus far. To address this problem, we have combined two complementary therapies: the antivascular drug DMXAA, which selectively inhibits tumor blood flow causing extensive hemorrhagic necrosis to the central zone (12), and antitumor antibodies conjugated to therapeutics, which selectively target tumor cells and can destroy the surviving outer zone of viable cells (13, 14). The mechanism of DMXAA action is mainly immunomodulatory and principally mediated by tumor necrosis factor-a induction, as demonstrated for both murine and human macrophages (15). The drug is currently in Phase

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3 The abbreviations used are: ADEPT, Antibody-directed Enzyme Prodrug Therapy; CPG2, carboxypeptidase G2; CEA, carcinoembryonic antigen; DMXAA, 5,6-dimethylxanthene-4-acetic acid; CMDA, 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid.

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I clinical trials and is showing significant biological activity by dynamic gadolinium-DTPA magnetic resonance imaging.4

We have shown that, although an optimal dose of DMXAA (27.5 mg/kg) causes necrosis of up to 95% of the tumor, it does not enhance survival. However, by combining DMXAA with an antibody-radioisotope conjugate (18.5 MBq of 131I-ASB7), which alone inhibits tumor growth for ~30 days, we have successfully eradicated colorectal xenografts in 85% of mice (14). Here, we investigate whether DMXAA will similarly enhance the therapeutic potential of ADEPT in the LS174T xenograft model, using F(ab')2 ASB7-CPG2 conjugate and the prodrug CMDA, which is activated to the drug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino] benzoic acid. We demonstrate that the use of DMXAA in combination with ADEPT, in addition to killing a large proportion of the tumor, doubles the concentration of antibody–enzyme conjugate retained in the tumor and significantly prolongs the tumor growth inhibition created by conventional ADEPT alone. Preliminary investigations indicate that the inhibition of blood flow produced by DMXAA can also increase prodrug retention within the tumor by 16-fold, depending on relative administration timing of the two drugs. Therapy experiments aimed at exploiting these increased tumor drug levels are ongoing.

MATERIALS AND METHODS

Drugs

DMXAA. DMXAA, provided by the Cancer Research Campaign (London, United Kingdom), was prepared in saline immediately prior to use and given as a single i.p. injection at 27.5 mg/kg. Previous experiments have shown this to be the optimal dose in the LS174T xenograft model, giving maximum tumor necrosis without toxicity (14).

CMDA. The prodrug CMDA was synthesized as described previously (16) and injected i.p. at a total dose of 1500 mg/kg. Prodrug was administered when circulating enzyme had fallen to between 0.1 and 0.2 units/ml in plasma, a level that did not create systemic toxicity in previous studies (10).

Antibody-Enzyme Conjugate

ASB7, a monoclonal anti-CEA antibody, and its fragments are in clinical use for cancer therapy within our department (17). The F(ab')2 fragment of ASB7 was conjugated to the bacterial enzyme CPG2, as described previously (18). The conjugate had an enzyme activity of ~120 units/mg, with 1 unit of activity defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmol min⁻¹ ml⁻¹ methotrexate in reaction mixture (19), and groups receiving the conjugate were given 25 units of CPG2 per mouse. For experiments involving 125I-F(ab')2 ASB7-CPG2, the conjugate was labeled with 125I using the chloramine T method.

Animal Studies

Xenograft. The human colon adenocarcinoma cell line LS174T was used to develop a xenograft model in the flank of female nude (nu/nu) mice. Subsequent passing was by s.c. implantation of small tumor pieces (~1 mm³). The tumor is a moderately differentiated CEA-producing adenocarcinoma with small glandular acini, which secretes no measurable CEA into the circulation (20). ASB7 gives positive staining for glandular luminal surface and cytoplasm. All mice used were aged 2–3 months and weighed 20–23 g at the initiation of the experiments.

Radiolabeled Conjugate Biodistribution. The effect of DMXAA administration on subsequent conjugate biodistribution in tumor and normal tissues was investigated by administering 20 μg 0.74 MBq of 125I-labeled conjugate to groups of four mice, which subsequently received either no further treatment or DMXAA at 20 h. At selected time points, the animals were bled, and liver, kidney, lung, spleen, colon, muscle, and tumor were removed for comparative activity assessment using a gamma counter (Wizard; Pharmacia, Milton Keynes, United Kingdom). Results were expressed as percentage injected dose per gram of tissue. Phosphor plate image analysis was performed on formalin-fixed sections of tumor and normal tissues.

Enzyme Biodistribution. The effect of DMXAA administration on active enzyme levels in tumor and normal tissues was investigated by the administration of conjugate containing 25 units of CPG2 to groups of four mice, which subsequently received either no further treatment or DMXAA at 20 h. At selected time points, the animals were bled, and liver, kidney, lung, spleen, and tumor were removed and assayed by high-performance liquid chromatography for CPG2 activity by the method described previously (21).

Prodrug Distribution. The effect of DMXAA on prodrug retention in tumor and normal tissues was investigated in groups of three mice that had received DMXAA at 6, 3, 2, or 1 h before or simultaneously with CMDA prodrug (single dose of 1200 mg/kg). Control mice received one dose of CMDA prodrug alone (1200 mg/kg). Mice were culled 1 h following CMDA administration; this allowed three half-lives, the half-life of the prodrug being ~20 min (10). All mice were bled, and liver, kidney, lung, spleen, and tumor were removed and assayed by high-performance liquid chromatography for prodrug concentration (10).

Therapy Studies. Experiments commenced when the tumors reached 0.1–0.2 cm³ and were in exponential growth, between 7 and 10 days after passing, using six mice per group. Different control groups included no treatment, antibody–enzyme conjugate plus DMXAA, and CMDA prodrug plus DMXAA. For the test group receiving the conventional ADEPT schedule, CMDA was given in three doses i.p. (500 mg/kg per dose) at 72, 84, and 96 h post conjugate. For ADEPT therapy groups combining DMXAA, the latter was administered at 20 h post conjugate, followed by the prodrug protocol above. Tumors were measured on the day of conjugate injection and on every subsequent 3rd or 4th day until tumor volume reached 2.0 cm³, when the mice were culled. The measurements were carried out in three dimensions (length, width, and height), and the tumor volume estimated as length × width × height/2 (22).

Statistics. For all biodistribution studies, the different treatment groups were compared using the Mann-Whitney U test. Comparison of survival between therapy groups was performed according to the Lee and Desu statistics (23). A P of <0.05 was considered significant here.

Toxicity Studies. Mice were weighed on the day of conjugate administration and on every 3rd or 4th day until the experiments were completed. Normal tissues were also examined histologically for signs of morphological change.

Histology Studies and Phosphor Plate Imaging. The effect of DMXAA on tumor morphology and 125I-labeled conjugate distribution was investigated by histochemistry and phosphor plate image analysis (autoradioluminography; Ref. 24). Using tumors from biodistribution studies, a series of 4-μm formalin-fixed paraffin sections were prepared from each of the four mice per group. Sections were exposed to phosphor storage plates and scanned with a Storm 860 phosphor image analyzer (Molecular Dynamics, Sevenoaks, United Kingdom), and the digitized images of labeled conjugate distribution were analyzed using ImageQuant software (Molecular Dynamics) and ImageJ software (National Institutes of Health). The measured activity was expressed as mean counts per pixel. Adjacent tissue sections were subsequently stained with H&E to relate the conjugate localization, with or without DMXAA treatment, to tumor morphology.

RESULTS

Effect of DMXAA on Biodistribution of Radiolabeled Antibody-Enzyme Conjugate. Previous experiments had shown that the conjugate achieved maximal tumor localization at ~20 h after administration (25). We, therefore, chose this time point to deliver a single optimal dose (27.5 mg/kg) of DMXAA and studied the effect of inhibiting tumor blood flow on subsequent conjugate biodistribution. Total conjugate levels in the tumor remained stable between 20 and 48 h post injection, and the addition of DMXAA at 20 h did not significantly alter this (P = 0.48; Fig. 1A). However, by 72 h, there was a significantly higher retention of conjugate within the tumor (P = 0.038) for those mice receiving DMXAA (Fig. 1B). The levels of conjugate localization in normal tissues remained unaffected by the additional administration of DMXAA (Fig. 1). This increase in tumor retention of conjugate following DMXAA and the greater homoge-
Effect of DMXAA on Biodistribution of Active CPG2 Enzyme. The levels of active enzyme (units/g) in tumor and normal tissues, with and without DMXAA given at 20 h post-conjugate administration, were investigated at the same time points chosen for studying the radiolabeled conjugate biodistribution. Again, the presence of DMXAA did not significantly alter the levels of CPG2 in normal tissues at 48 or 72 h post-conjugate delivery (28 and 52 h post-DMXAA administration). Tumor enzyme levels also remained unchanged ($P = 0.51$) at 48 h (Table 1). However, in agreement with the results for radiolabeled conjugate, DMXAA significantly enhanced the retention of active enzyme within the tumor by 72 h post-antibody-CPG2 administration ($P = 0.025$).

Effect of DMXAA on Prodrug Localization. Fig. 3 shows the amount of prodrug retained in the tumor by 1 h after administration, when prodrug was given at selected times following DMXAA. Results are compared with a control group (Fig. 3, C) receiving prodrug alone. Simultaneous administration of prodrug and DMXAA (Fig. 3, D) did not alter tumor uptake compared with controls ($P = 0.83$). There was a dramatic increase in tumor retention of prodrug when given at 4 h after the DMXAA, which was 16 times higher than the control value (means of 44.8 and 2.8 μg/g tumor, respectively) and significantly higher than for any other time point studied ($P = 0.049$). Although there also appeared to be a trend toward increased tumor retention of prodrug, when it was given at 1, 2, and 3 h after DMXAA, there was no significant difference between any of these groups and the control mice ($P = 0.13, 0.51$, and 0.83, respectively). However, they were all significantly higher than the group receiving prodrug at 6 h after DMXAA ($P = 0.049$). The latter group did not differ significantly from the control or joint administration groups ($P = 0.126, 0.51$). The addition of DMXAA did not significantly alter the prodrug levels in any normal tissues studied ($P > 0.5$).

Effect of DMXAA on ADEPT Therapy. Fig. 4 shows the mean tumor growth over time of LS174T xenografts, following a range of different treatment regimes. We have previously demonstrated that DMXAA, CMDA prodrug, and antibody-enzyme conjugate had no significant effect on tumor growth when they were given alone (14, 25). These experiments showed that the same was true for conjugate plus DMXAA (data not shown) and prodrug plus DMXAA (Fig. 4). For conventional ADEPT, the prodrug was given as three doses of 500 mg/kg over 12 h, commencing when plasma enzyme levels had fallen to between 0.1 and 0.2 units/ml. This produced significant tumor growth inhibition, compared with all control groups. However, the additional administration of DMXAA at 20 h post-conjugate injection, followed by prodrug at the normal time of 72 h, significantly enhanced the therapeutic effect produced by the conventional ADEPT treatment (Fig. 4).

Toxicity Studies. Fig. 5 illustrates the toxicity of the various therapy regimes, as reflected by changes in weight over time, the mean values from six mice per group being expressed as a percentage of their pretreatment values and presented until these original values were regained or the group had been culled. For the untreated control group and those receiving CMDA or DMXAA either alone (data not shown) or combined, the weights continued to rise throughout the experiment. The greatest weight loss was seen in mice receiving conventional ADEPT, where the mean value dropped to 81% of starting weight at 11 days post conjugate injection. A less pronounced weight loss and a more rapid recovery to pretreatment values was seen in the group receiving combined DMXAA and ADEPT, with a low of 91% at 7 days. Histological studies revealed no evidence of normal tissue damage following any of the treatment regimes.

**DISCUSSION**

This study has shown that combining ADEPT, which targets tumor cells, with an antivasculcar strategy can significantly enhance the therapeutic effect of either treatment alone. A similar enhancement of radioimmunotherapy was obtained by combining either flavone acetic acid or its more potent analogue, DMXAA, with 18.5 MBq of $^{131}$I-ASB7 (13, 14). Scheduling was crucial because the radioantibody had to reach maximum tumor accumulation at 48 h before initiation of drug-induced blood flow inhibition. Subsequent tumor localization of antibody conjugate can only occur via the surviving blood vessels at the viable rim. Ongoing work combining radioimmunotherapy with
the tubulin binding agent combretastatin 4-A, which also induces rapid vascular shutdown like DMXAA (11), is showing similar results. Currently, the situation is even more complex because both an antibody-enzyme conjugate and a prodrug are now involved (3). We have demonstrated by gamma counting, phosphor imaging, and active enzyme assay that administration of DMXAA at the time of peak antibody-CPG2 conjugate (20 h) produced a 2-fold increase in conjugate levels within the tumor at the time of prodrug administration (72 h).

A major advantage of the ADEPT system is that the antibody-enzyme conjugate can be targeted to the tumor and allowed to clear from normal tissues before the prodrug is administered, thus generating the cytotoxic drug selectively at the tumor site and reducing systemic toxicity. However, by the time the CPG2 in the plasma has fallen to a safe level, the amount of conjugate localized in the tumor has also decreased and may be insufficient to produce a prolonged therapeutic effect (25). There are several ways of overcoming this problem. One is to use a three-phase system, in which a galactosylated anti-CPG2 antibody is used to inactivate and clear the circulating enzyme (25). This allows the prodrug to be given at 20 h instead of 72 h post-conjugate administration, when enzyme levels in the tumor are at their peak and there is the potential for greater drug activation and enhanced therapeutic effect. An alternative strategy, which we are currently investigating, is to use a fusion protein composed of an anti-CEA single chain Fv fragment and an enzyme (MFE:CPG2). This clears more rapidly from the circulation to give improved tumor:blood ratios, allowing earlier administration of prodrug while enzyme levels within the tumor are at a peak (26).

Here, we demonstrate a third concept: that conventional ADEPT, as used in clinical trials, can be significantly enhanced by addition of an antivascular agent, which can both

Table 1 Active enzyme levels in tumor over time post-conjugate administration, with and without DMXAA

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<th>Time post-conjugate administration</th>
<th>Without DMXAA</th>
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<tr>
<td>20 h</td>
<td>1.86 ± 0.02</td>
<td>1.18 ± 0.5</td>
<td>1.58 ± 0.2</td>
<td>0.53 ± 0.09</td>
<td>0.99 ± 0.08</td>
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DMXAA was administered at 20 h post-conjugate administration. Values represent means ± SD, calculated from values for four mice per group.

Fig. 2. Phosphor image and histological appearance (H&E) of 125I-F(ab’)2 A5B7-CPG2 localization in sections of LS174T tumor at 72 h postadministration, without (top) and with (bottom) 27.5 mg/kg DMXAA given at 20 h. H&E sections correspond to the leftmost phosphor images.

Fig. 3. LS174T tumor levels of prodrug at 1 h postadministration of a single dose of 1200 mg/kg, without (C) or with (0–6) combined administration of DMXAA (27.5 mg/kg) at times from 0 to 6 h prior to prodrug. Columns, means of three animals per group, bars, SE.

cause extensive tumor necrosis and also retain high levels of conjugate within the tumor mass but not in normal tissues. This may be purely an additive effect of the two complementary therapies. Alternatively, retention of increased tumor levels of antibody-enzyme conjugate at the time of prodrug administration, following blood vessel destruction by DMXAA, may allow conversion of more prodrug to active drug and lead to enhanced tumor growth inhibition. The mode of action for the combined therapies is under investigation. Either way, this is potentially the most effective strategy of the three, because DMXAA is killing those areas of tumor either not reached by, or relatively resistant to, antibody-targeted therapies (3).

A potential problem of combined therapies is increased toxicity, but this has not proved to be the case for DMXAA and ADEPT (Fig. 5). Indeed, it appears from this and our previous studies (14) that DMXAA may actually reduce the level of toxicity seen after antibody-directed therapies. Similarly, there was no increase in mortality when DMXAA was combined with melphalan (27). It has been shown that tumor necrosis factor-α, which is induced by the action of DMXAA, can reduce the damaging effects of ionizing radiation on bone marrow progenitor cells (28) and that similar protection may be occurring here when the antivascular agent is combined with chemotherapy.

Attempts to trap the prodrug within the tumor mass by combined treatment with DMXAA have produced interesting preliminary results (Fig. 3). A dramatic increase in tumor prodrug retention was created by preadministration of DMXAA, but the effective time window was found to be very narrow. Giving the two drugs simultaneously did not enhance prodrug retention, whereas giving the DMXAA at 1, 2, or 3 h before the prodrug appeared to slightly increase the level of CMDA in the tumor, although not significantly higher than controls for this small sample size. However, by delaying the prodrug delivery until 4 h post DMXAA, we produced a 16-fold increase in tumor retention of prodrug, which was significantly higher than any other group studied. By 6 h, the effect was reversed: although results did not differ from the control group, a significant reduction in CMDA uptake was found, compared to groups given DMXAA at 1, 2, and 3 h prior to prodrug. Tumor histology at the same experimental time points showed that these results correlated with the time course of vascular shut-down induced by the antivascular agent. Although signs of coagulation were visible as early as 1 h following DMXAA, it was between 4 and 6 h that blood vessel occlusion progressed to complete vessel breakdown with hemorrhaging into the tumor. Therefore, the critical time for allowing prodrug to enter the tumor, while inhibiting its subsequent escape by DMXAA-induced blood vessel destruction, lies between 4 and 5 h post DMXAA for the LS174T xenograft. The fact that all of the tumors examined at the 4-h time point showed a dramatic increase in prodrug level indicates that timing is precise for this model system.

In support of these findings, predadministration of DMXAA has been shown to decrease tumor clearance and augment the antitumor activity of melphalan in a mouse mammary tumor (27). They also demonstrated a time-dependent relationship between the two drugs, although this was slightly shorter for the mouse tumor than we observed in the colorectal xenograft: tumor clearance of melphalan started to fall at 3 h after DMXAA, and direct measurements showed a 70–75% inhibition of blood flow by 4 h post-DMXAA administration. Whether the entrapped prodrug in this study is available for conversion to active drug by prelocalized antibody-enzyme conjugate is currently under investigation. If favorable results are obtained, future therapy experiments will include the administration of DMXAA at 68 h post-conjugate administration and 4 h pre-prodrug administration and investigate whether the subsequent 16-fold increase in prodrug levels can be translated into enhanced ADEPT treatment. This will determine whether trapping conjugate or prodrug within the tumor will give the greater therapeutic advantage. Earlier work using a CPG2 ADEPT system in a choriocarcinoma xenograft model indicated that the amount of available prodrug rather than conjugate was the limiting factor for therapy because a single enzyme molecule could activate many drug molecules (29). The antibody-enzyme conjugate must, however, be at a site in the tumor which is accessible for prodrug activation. This is of particular relevance to situations such as our current clinical trial, which involves the novel bis-iodo phenol mustard drug ZD2767 with an extremely short chemical half-life of ~2 min in plasma (8).

In conclusion, the use of a drug which selectively inhibits tumor blood flow and creates extensive necrosis can significantly potentiate the antitumor action of ADEPT. This effect may be created by one of three mechanisms: (a) a simple additive killing of the tumor center by
DMXAA and the rim by ADEPT; (b) the increased retention of ADEPT components within the tumor by DMXAA activity; or (c) a combination of (a) and (b). The implication from this work is that a greater understanding of both drug action and tumor biology, leading to improved synergistic or complementary combinations of different therapeutic modalities, will provide the best potential for successful cancer therapy. Attacking the tumor vasculature, where the destruction of one capillary can lead to the death of many cancer cells, appears to be an effective treatment for use in combination with both radioimmunotherapy and ADEPT, and many other drugs and biological response modifiers have potential for use in this field.

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Enhancement of Antibody-directed Enzyme Prodrug Therapy in Colorectal Xenografts by an Antivascular Agent

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