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Preclinical report

Methotrexate–albumin conjugate causes tumor growth delay in Dunning R3327 HI prostate cancer-bearing rats

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Based on the rationale of a preferred albumin uptake by tumors, conjugates comprising of rat serum albumin (RSA) as a drug carrier and of methotrexate (MTX) as chemotherapeutic drug were prepared. For a comparative study of MTX–RSA and MTX we chose a slow growing Dunning R3327 HI prostate cancer model. In a radiopharmacologic study blood kinetics and the tumor and organ distribution pattern of residualizingly labeled MTX–RSA were determined, and were found to be similar to that of residualizingly labeled RSA. The MTD was established for Copenhagen rats at a total four injections of 2 mg/kg MTX or MTX–RSA administered at days 0, 4, 8 and 12. Tumor volume measurements and tumor removal showed a small non-significant growth delay in the MTX treatment group, suggesting MTX resistance for the Dunning R3327 HI prostate carcinoma. In contrast, treatment with MTX–RSA resulted in a significant (50%) growth inhibition of the Dunning R3327 HI tumor. The cellular mechanisms responsible for MTX resistance in Dunning HI tumor cells is not known. The improved therapeutic effects seen during MTX–RSA treatment in this slow growing adenocarcinoma might be a result of prolonged tumor exposure time and an altered cellular uptake by a lysosomal route. MTX–albumin conjugates have shown antitumor activity exceeding that of MTX in several tumor xenografts in nude mice, including human prostate cancer. The recently initiated clinical development of MTX–human serum albumin will be continued and cancer of the prostate will be included as a potential target tumor during further clinical phase II testing. [© 1999 Lippincott Williams & Wilkins.]

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Introduction

Plasma proteins constitute the major transportable nitrogen reserve in vertebrates.[1] Hence, proliferating cells, especially tumor cells, possess elaborate mechanisms to tap this supply. After endocytosis, plasma proteins will be delivered to lysosomes. Lysosomal proteases degrade plasma proteins to their constituent amino acids, which might then serve as a source for tumor nitrogen and energy.[2] Recent data suggest that, apart from the well-known functions as a transport protein or keeping up osmotic pressure, albumin plays an important role as a nutrient for proliferating tumors. Animal experiments and studies in man confirmed an enhanced uptake of albumin by tumors, if residualizing radiolabels were used for mapping degradation sites.[3–6]

Based on the rationale of a preferred albumin uptake by tumors, conjugates comprising of albumin as a drug carrier and of methotrexate (MTX) as chemotherapeutic drug were prepared. Interestingly, drug conjugates bearing multiple MTX molecules per albumin were rapidly removed from circulation and predominately trapped by the monocyte-macrophage system of the liver. Tumor targeting of these highly loaded conjugates was poor.[7] Only albumin conjugates derivatized with MTX at a ratio close to 1:1 offered the same favorable distribution pattern with a high tumor uptake rate as native albumin.[8] A treatment study in rats bearing a MTX-sensitive rapidly proliferating

Walker-256 carcinoma demonstrated the chemotherapeutic efficacy of a MTX-rat serum albumin (RSA) conjugate.[9] For this comparative treatment study of MTX-RSA and MTX, we chose the Dunning R 3327 HI prostate cancer with slow proliferation kinetics imitating the situation in man more closely than a Walker-256 carcinoma.[10-12]

Material and methods

Preparation of the MTX–RSA conjugates

RSA (65 871 Da) and MTX (amethopterin; 454.45 Da) were purchased from Sigma (Deisenhofen, Germany). Hydroxysuccinimide (HSI), *N,N'*-dicyclohexylcarbodiimide (DCC) and Trypan blue were delivered by Aldrich (Steinheim, Germany). For separation of the compounds, Centricon ultrafiltration units from Amicon (Witten, Germany) were used. Conjugates from albumin and MTX were prepared by a recently published technique.[7] In short, DCC was used for the activation of MTX. A typical reagent mixture contained MTX at 20 mg/ml, dissolved in DMF. To about 1 ml of this solution, 14 mg DCC and 50 mg of HSI were added. The formation of the activated methotrexate (MTX-SE) was completed after 12 h. MTX-SE was slowly added to a solution of 20 mg RSA/ml (dissolved in 0.13 molar sodium phosphate buffer, pH 7.4). The remaining low molecular weight reagents were separated from MTX-RSA by ultrafiltration using a membrane with an exclusion size of 30 000 Da. The respective binding rates of MTX to RSA were calculated after a photometric determination of the amount of unbound MTX in the filtrate. The concentration of MTX was determined by absorption at 370 nm in sodium bicarbonate buffer, 0.17 M, pH 8.5.

Preparation of a residualizingly radiolabeled MTX–RSA

For the determination of the plasma levels and the distribution patterns of MTX-RSA in tumor-bearing rats, the conjugates were tagged with a residualizing [^{111}In]DTPA label to RSA. The coupling of DTPA to RSA or MTX-RSA was carried out using DCC and HSI for DTPA activation. Unbound reagents were separated by washing with 0.17 M sodium bicarbonate solution in a Centricon ultrafiltration C30 unit. For radiolabeling 185 MBq $^{111}InCl_3$ (5 mCi) was mixed with 5 μl of a 0.2 M sodium citrate solution and then added to DTPA-MTX-RSA dissolved in 1 ml 0.17 M sodium bicarbonate. Unbound tracer was separated by a Centricon ultrafiltration C30 unit. The labeling yield was about 97%. An analytical HPLC control run of the purified [^{111}In]DTPA-MTX-RSA tracers showed impurities of below 1%. The loading rate of the DTPA label was also adjusted to a molar ratio of about 1:1 with RSA. The conjugation technique did not lead to the formation of multimers of albumin. The HPLC profiles of radiolabeled [^{111}In]DTPA-MTX-RSA matched with the profile of RSA that had been obtained before the chemical modification by MTX or DTPA conjugation (further details in Stehle *et al.*[7]).

The tumor model

WF Dunning isolated and classified a papillary adenocarcinoma of the prostate during the autopsy of a male Copenhagen (COP) rat in 1963.[10] From this primary Dunning prostate carcinoma a variety of sublines were grown, differing in growth characteristics and in hormone receptor status.[13-17] Up to 90% of the original Dunning tumor cells required androgens for growth, the remaining 10% were hormone independent (HI). For our study we chose the hormone-independent R3327 HI subline. Vital tumor fragments had initially been obtained by generosity of JT Isaacs (John Hopkins University, Baltimore, MD). This subline was histologically classified as a moderately differentiated mucin-producing adenocarcinoma with a low metastatic potential.[15] Tumor fragments were stored deep frozen (−196°C) in Hank's balanced salt solution enriched with 20% DMSO. The fragments were passaged by s.c. implantation to male COP donor rats. After 8 weeks the R3327 HI tumors had reached a tumor weight of 2-3 g. The tumor-bearing rat was killed by overanesthesia with CO_2 to prepare tumor fragments. The tumors were carefully cut into small fragments of about 3 mm in diameter. These fragments were transplanted s.c. after a small incision at the left hind leg of the COP rats. After a period of 5 weeks these tumors had reached a size of about 1 cm^3.

Figure 1 shows the growths kinetics in rats bearing a s.c. implanted Dunning R3327 HI tumor compared to rats with fragments of s.c. implanted ovarian-342 tumor or to rats with an i.v. seeded Walker-256 carcinoma. Tumor volume doubling times were determined at the beginning of exponential growth. The Walker-256 carcinoma grew rapidly with a doubling time of 44 h. This value was comparable with a report in literature of 42.8 h.[18] The tumor volume doubling time of the ovarian-342 tumor was 5.2 days and of the R3327 HI tumor was 8.5 days. The Walker-256 carcinoma and the ovarian-342 rat tumor

models used in this study have been described in detail elsewhere.[5]

Male COP rats, weighing 250–300 g, were obtained from Olac (Blackthorn, UK). The rats were kept under standard living conditions in the Central Animal Laboratories of the German Cancer Research Center, Heidelberg. The animal experiments had been approved by the German Federal Government (Regierungspräsidium Karlsruhe AZ 72/1994 and 100/1995 to GS). After tumor fragment implantation at the left hind leg of the COP rats, the tumor radii were measured with calipers and the tumor volume was estimated by using the formula of a rotating ellipsoid $V = 4/3(a \times b^2)$, a is the longer radius. The tumor volume based on a rotating ellipsoid correlated with the weight of 40 Dunning R3327 HI tumors with a Bravais–Pearson coefficient of 0.97, $p < 0.001$.

Radiokinetic studies

Considerable information on the pharmacokinetic properties of MTX-RSA is available for Sprague-Dawley (SD) rats bearing the Walker-256 carcinoma.[8] To confirm these data for Dunning HI-bearing COP rats, we measured the blood levels and tissue and tumor distribution of a residual radiolabeled MTX-RSA in a 72 h single-dose study. Five rats received i.v. injections of [^{111}In]DTPA-MTX-RSA (100 μCi=3.7 MBq; 100 μg of the conjugate), dissolved in 300 μl bicarbonate buffer (0.17 M, pH 8.4) into a lateral tail vein. Throughout the experiments the rats were anesthetized by a mixture of halothane, N_2O and O_2 (1.5/60/38%). Blood samples (20 μl per sample) were drawn after incising the tail tip at 1, 4, 8, 24, 48 and 72 h after tracer administration triplicate. A reference curve was prepared using triplicate serial dilutions of the tracer substance. After each blood sampling procedure the initial standard tracer dilution was recounted to adjust for the radioactive decay of ^{111}In. The equation 'blood volume=0.06 × body weight + 0.77' was used to estimate the blood volume of the rats.[19] From these data the percentage of the injected radioactivity present in the blood at different times was calculated. The blood loss of the animals was less than 1 ml or below 5% of the respective total blood volume. After the final blood sampling the five animals were sacrificed 72 h post-injection, and the organs and the tumor were removed. After determination of the weight, the radioactivity of the samples was measured in a γ-counter. The results are expressed as percent of radioactivity uptake per organ initially injected to the animal.

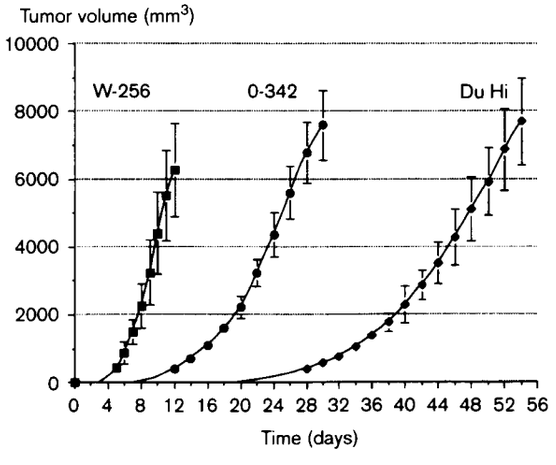

Figure 1. Tumor volume and growth kinetics of an i.v. seeded Walker-256 carcinoma (W-256) in SD rats, an ovarian-342 (O-342) tumor implanted as a s.c. fragment to female BD IX rats and of a Dunning R3327 HI prostate carcinoma implanted as a s.c. fragment to male COP rats (n=10 for each group, for further details see Materials and methods).

Treatment study design

Twenty COP rats were used to determine the dose-limiting toxicity (DLT). The first 10 rats received repeated injections of 2 mg MTX/kg body weight, the remaining 10 rats received 2 mg MTX-RSA/kg body weight. All rats were randomly allocated to the MTX or MTX-RSA group. MTX or MTX-RSA were administered every 4 days until occurrence of DLT. The maximum tolerated dose (MTD) was defined as DLT minus one injection. Then, 30 tumor-bearing rats were randomly distributed among the two treatment groups (MTX or MTX-RSA) and a control group receiving saline injections. Treatment started after the tumor volumes of the s.c. transplanted Dunning HI fragments had grown to tumor volumes of 600–800 mm^3, usually after 5–6 weeks after tumor seeding. All rats were checked for signs of toxicity daily. The body weight and the tumor volumes were determined every second day. The study was terminated after the tumor burden of the control group had reached a critical size following the guidelines of the German Animal Welfare Regulations 24 days after treatment initiation. After a final determination of the tumor volumes, all animals were sacrificed, and the tumors removed and the tumor weights were recorded. Tumor volumes and weights of the three groups were statistically evaluated using the t-test.

Results

Radiokinetic distribution of residual radiolabeled MTX–RSA

Five COP rats bearing a Dunning HI adenocarcinoma received injections of radiolabeled MTX-RSA to confirm the prolonged plasma presence and the characteristic tissue distribution pattern of the drug conjugate known from studies in Walker-256 carcinoma-bearing SD rats or ovarian-342 tumor bearing Burdette-Duckrey rats (BD IX). The respective tracer presence in plasma is shown in Table 1 over 72 h. After 24 h about 20% of the initially injected dose was still detected in blood, declining to 10.4% after 48 h and 6.1% after 72 h. The tissue distribution of the MTX-RSA tracer was studied after 72 h and showed a low uptake rate of well below 10% of the injected doses by liver and kidney (Table 2). This organ distribution pattern did not differ from our previous experience with the MTX-RSA conjugate in other rat models. The tumor took up 1.6% of the injected activity (tumor mass 0.9% of the body weight) after 72 h. The tumor uptake rate of the MTX-RSA tracer exceeded the background uptake (carcass after removal of organs and tumors) by 2- to 3-fold.

Table 1. Pharmacokinetics of residually radiolabeled MTX–RSA in blood

Time (h)	MTX–RSA (% ID)	
	Mean	SD
1	83.2	2.5
4	61.1	1.7
8	42.0	0.8
24	20.1	1.1
48	10.4	0.9
72	6.1	0.3

Percentages of tracer presence in circulation are shown, based on the initially injected amount of [^{111}In]DTPA–MTX–RSA (% ID) in COP rats bearing a Dunning HI prostate adenocarcinoma over a period of 72 h (3.7 MBq tracer was administered; tumors sharing about 1% of the body weight; n=5).

Table 2. Tissue and tumor uptake rates (%) of residual [^{111}In]DTPA–MTX–RSA in five COP rats bearing a Dunning HI adenocarcinoma (the organs were removed after 72 h)

	MTX–RSA	
	Mean	SD
Blood	6.1	0.3
Liver	8.4	0.2
Kidneys	6.2	0.4
Spleen	0.7	0.1
Lung	1.1	0.2
Intestines	3.9	0.2
Tumor	1.6	0.1

Determination of the DLT

Before conducting a comparative treatment trial with MTX or the macromolecular prodrug conjugate MTX-RSA, DLT and MTD for both compounds were established. Twenty COP rats received five injections of 2 mg MTX/kg or of 2 mg MTX/kg conjugated to RSA (2 mg MTX-RSA/kg). The dose was adapted from a previous treatment trial with Walker-256 carcinoma in which a cumulative dose of 6 mg MTX/kg administered on days 0, 2 and 7 had been well tolerated and achieved therapeutic responses.[9] Based on the long *in vivo* half-life of the RSA conjugate treatment, intervals of 4 days were chosen to prevent drug accumulation. All injections were well tolerated. A few days after the fifth injection both groups showed signs of typical MTX toxicity with mucositis, diarrhea and shaggy fur. The side effects occurred in the MTX-RSA group compared to MTX group with a delay of a few days. The MTD was established at four injections on days 0, 4, 8 and 12 for the treatment experiment.

Treatment trial

Three groups were formed, each with 10 randomly allocated Dunning HI-bearing COP rats. The rats of the MTX group received i.v. injections of 2 mg MTX/kg on days 0, 4, 8 and 12, the rats of the MTX-RSA group 2 mg MTX-RSA, and the rats of the control group received saline. During the study period of 24 days no treatment-induced toxicity or weight loss was observed (Figure 2). Tumor volume determination revealed a slight tumor growth retardation for MTX during the treatment period. A more pronounced effect was observed for MTX-RSA. The tumor volumes of the control group had reached a mean value of about 9000 mm^3, the MTX group of 7900 mm^3 and the MTX-RSA group of 4800 mm^3 after 24 days. These results were confirmed after tumor removal by comparing the tumor weight (Figure 3). No significant growth retarding effect was observed between the control group and the MTX group, whereas MTX-RSA

treatment resulted in a significant tumor growth delay compared to the control group or the MTX group (*t*-test; $p < 0.001$).

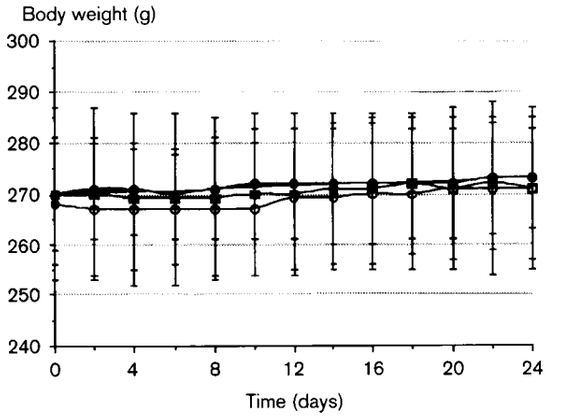

Figure 2. Body weight distribution as a measure for treatment side effects after the repeated administration of (●) 2 mg MTX/kg, (○) 2 mg MTX–RSA/kg or (■) untreated control to rats bearing a Dunning R3327 HI prostate carcinoma (n=10 rats for each group).

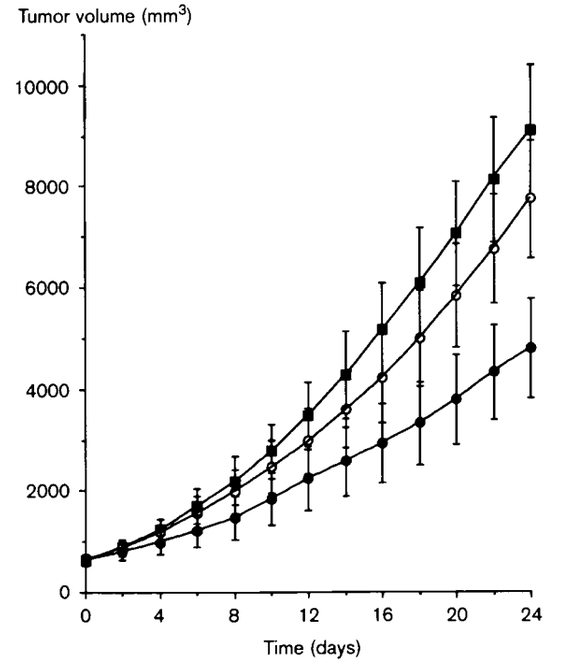

Figure 3. Tumor volume growth kinetics after the repeated administration of (○) 2 mg MTX/kg, (●) 2 mg MTX–RSA/kg or in (■) untreated rats bearing a Dunning R3327 HI prostate carcinoma (n=10 rats for each group, mean values ± SD, for details on volume determination see Materials and methods).

Discussion

Radiokinetic distribution of residualizingly radiolabeled MTX–RSA

MTX conjugated to serum albumin in a molar ratio close to 1:1 is a novel approach to improve the therapeutic efficacy of MTX. The pro-drug conjugate MTX-RSA enjoyed a considerably prolonged tumor exposure time in SD rats compared to MTX in a radiokinetic study.[8] In a previous treatment study in SD rats bearing a fast growing Walker-256 carcinoma we have shown that MTX-RSA was therapeutically slightly more efficient than MTX, a combination of both showed a potentiated therapeutic effect. However, a shortcoming in this design was the rapid tumor growth of the rodent Walker-256 carcinoma (tumor volume doubling time 44 h, Figure 1). Chemotherapy with MTX-RSA may benefit from long tumor exposure times, tumor accumulation of albumin and different uptake mechanisms by cancer cells. Repeated injections of the conjugate and long-term treatment is necessary to exert an optimal antineoplastic effect. For that purpose we chose a slow growing Dunning R3327 HI prostate cancer model for which we established a tumor volume doubling time of 8.5 days. The R3327 HI subline was histologically classified as a moderately differentiated mucin-producing adenocar-

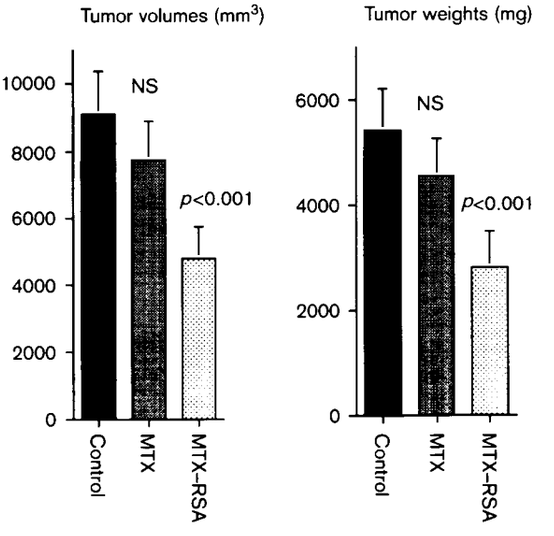

Figure 4. Comparison of tumor volumes (mm^3) and tumor weight (mg) after the repeated administration of either 2 mg MTX/kg, 2 mg MTX–RSA/kg or in untreated rats bearing a Dunning R3327 HI prostate carcinoma at the end of the treatment period after 24 days (n=10 rats for each group, mean values ± SD, *t*-test, $p < 0.0001$).

cinoma with a low metastatic potential.[15] Recently, increased MTX resistance has been reported for the hormone insensitive Dunning AT1 and MatLu cell lines in a cell culture study, and it has been suggested that this kind of multidrug resistance was P-glycoprotein mediated.[20] To our knowledge there is no evidence available whether this might apply to the HI subline as well.

Before conducting a comparative treatment experiment in the R3327 HI we studied the blood kinetics, and the tissue and tumor distribution on the MTX-RSA drug conjugate. Based on previous experience we chose a residualizing protein radiolabel based on [^{111}In]DTPA.[5] Residualizing protein labels are widely used for mapping protein degradation sites, as the radiolabel remains trapped at the lysosomal degradation sites of the protein for several days reflecting protein turnover.[21]

The blood kinetics of the radiolabeled MTX-RSA conjugate in R3327 HI-bearing COP rats was comparable to the data for radiolabeled RSA in SD and BD IX rats. After 24 h 20.1% and after 72 h 6.1% of the initially injected amount MTX-RSA tracer was measured in blood (SD rats 18.4%, 7.1%; BD IX rats 19.4%, 5.7%).[5]

The liver uptake rate of the MTX-RSA conjugate was 8.4% of the injected dose and not decisively increased compared to RSA (7.2% after 72 h in BD IX rats; 6.7% in SD rats).[5] A much higher liver uptake (more than 60% of the injected dose) mediated mostly by macrophage scavenger receptors had been observed, if MTX-RSA conjugates had been denatured during conjugation procedures.[7] The tumor uptake rate of the MTX-RSA tracer exceeded the background uptake by 2- to 3-fold. However, a correct determination of the specific tracer uptake rates for this tumor was not possible. The histologic examination of the Dunning HI tumors revealed mostly glandular acini filled with massive amounts of PAS-positive mucous secretions surrounded by a comparatively small amount of tumor cells.

Determination of the MTD and treatment trial

Before conducting a treatment study we established the MTD for MTX and MTX-RSA at 2 mg/kg MTX or MTX-RSA injected at days 0, 4, 8 and 12. A fifth additional injection on day 16 caused severe MTX-induced toxicity in all rats. The treatment with the MTD was well tolerated by all rats, no side effects were observed and the body weight did not decline during the treatment experiments. Tumor volume measurements and tumor removal showed a small non-significant growth delay in the MTX treatment group, suggesting MTX resistance for the Dunning R3327 HI prostate carcinoma. In contrast, treatment with MTX-RSA resulted in a significant (50%) growth inhibition of the Dunning R3327 HI tumor, revealing moderate sensitivity. The cellular mechanisms responsible for MTX resistance in Dunning HI tumor cells are not known. The improved therapeutic effects seen during MTX-RSA treatment in this slow-growing moderately differentiated adenocarcinoma might be a combined result of prolonged tumor exposure time and an altered cellular uptake route via the lysosomal system. MTX-albumin conjugates have shown antitumor activity exceeding that of MTX in several human tumor xenografts in nude mice, including human prostate cancer.[22] In a recently completed phase I clinical trial of MTX-HSA in cancer patients we observed prolonged tumor responses in three patients (two kidney carcinoma and one pleural mesothelioma), two of which are still ongoing after more that 28 months under a continuing MTX-HSA treatment.[23] The clinical development of MTX-HSA will be continued and cancer of the prostate will be included as a potential target tumor during clinical phase II testing.

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