Original Paper

Combined Effects of Doxorubicin and Temozolomide in Cultured Glioblastoma Cells

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ABSTRACT: The oncological field benefits of extensive medical research and various types of cancer notice improvements, however glioblastoma multiforme remains one of the deadliest cancers in humans with virtually no advance in survival and clinical outcome. Temozolomide, the FDA approved drug for glioblastoma, faces numerous challenges such as resistance and side effects. To overcome these challenges, many combination therapies are currently studied. The present study analyses the effects of temozolomide in combination with doxorubicin on a glioblastoma cell line. Our results showed that both drugs displayed a cytotoxic effect on the studied cells in single administration (55% for 100μM temozolomide at 14 days, 53% for 100μM doxorubicin at 14 days), but without a synergistic effect in dual therapy. Although the results failed to produce the expected effect, they propose new research perspectives in the future.

KEYWORDS: *Glioblastoma, doxorubicin, temozolomide, cytotoxicity.*

Introduction

Tumours of the central nervous system (CNS) are a diverse group of neoplasias with a grave effect on patients' clinical presentation, evolution and wellbeing, at the pinnacle of which stands glioblastoma multiforme (GBM).

Glioblastoma multiforme, a grade IV neoplasm, represents one of the most frequently diagnosed malignant intracranial tumour (ICT) and one of the most malignant types of brain cancer [1,2].

Its aggressive nature is in part because of the countless mutations that GBM cells acquire rendering resistance to therapies and giving rise to numerous questions regarding the clinical importance of certain mutations and therapeutical possibilities of treating this disease [2,3].

Resistance to current standard of care is either innate or by adaptive pathways following the exposure to radiotherapy or chemotherapy [4].

Other features responsible for GBM's aggressive behaviour are: high proliferation rate, invasiveness (which hinders the possibility to obtain complete neurosurgical resection), high vascularization consisting of vessels resembling the renal glomeruli, high rates of recurrence and, in rare cases, the metastatic ability which is rather unique amongst other CNS tumours (lungs, liver, bones, pancreas, skin) [2,3,5].

GBM overall survival is approximately 14 months and it is achievable with surgical excision, radiotherapy, concomitant and adjuvant chemotherapy [6].

Survival depends on numerous factors such as: tumour's location, the ability to perform a complete resection, chemotherapy administration and Karnofsky performance status (KPS), but nevertheless the prognosis is grim [7].

Because of all of the features GBM possess, it is considered a dreaded, incurable disease, for which advancements in the better understanding of its pathological pathways and effective drug combination are most necessary.

The word multiforme refers to the heterogeneity both genetically and phenotypically, that result in inter-and intra-tumour heterogeneity which confer an unpredictable response to different types of therapies, hence the difficulty to obtain a positive response to single therapy.

In addition, another impediment that anti-GBM therapy might face is the limited number of drugs that are able to cross the dual blood-brain barrier (BBB)/blood brain tumour barrier (BBTB) and reach the targeted tissue in appropriate dosage [3].

Temozolomide (TMZ), the worldwide used drug to treat GBM, has a lipophilic nature that allows the passage of BBB, thus inducing cellular death in GBM. It is orally bioavailable, but can also be administered intravenously [8].

Treatment response and patient prognosis are dependent on the genetic alterations.

The following mutations exhibit a poor prognosis: epidermal growth factor mutation (EGFRvIII) most commonly observed in primary GBM, mutation in alpha-thalassemia/mental retardation syndrome X-linked gene (ATRX)-

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most commonly in secondary GBM, mutation in phosphatase and tensin homolog (PTEN)-primary GBM and ribosomal protein S6 (RPS6) is linked to a poor prognosis [4].

O-6-methylguanine-DNAmethyltransferase (MGMT) is an enzyme responsible for DNA repair in cancerous cells when exposed to temozolomide (TMZ).

Methylation of the gene encoding this enzyme makes the GBM cells sensitive to TMZ offering a better prognosis and improving patient survival with approximately 9 months [3].

However, treatment resistance to both radiotherapy and chemotherapy is almost a certitude in GBM [9].

A multitude of genetic, epigenetic and tumour environmental factors play a role in developing resistance in GBM cells, including the ones that are sensitive in the beginning.

Treatment response to TMZ is dependent on the MGMT methylation status.

Other mechanisms that induce treatment resistance are: deregulated signaling pathways (sonic hedgehog, protein kinase C, B-cell lymphoma 2), CD133+glioblastoma stem cells (GSCs) that increase the expression of MGMT and other proteins which exert an anti-apoptotic effect, DNA mismatch repair (MMR), enhanced base excision repair (BER), hypoxia areas that harbour GBM cells resistant to radiotherapy, macrophages that are recruited by the GSC and secret immunosuppressive factors such as interleukin-10 and transforming growth factor beta 1 [3,4].

A new approach to handling the problematic therapeutical management of GBM and other aggressive tumours is testing the response to combination therapy.

In *vitro* and in *vivo* studies showed that combination therapies should offer a few advantages such as: reducing the risk of developing resistance, obtaining a synergistic or additive effect and reducing the risk of adverse effects by reducing the dose of the therapeutical agents [4,10,11].

Many deregulated pathways are responsible for maintenance of GSC pool and subsequent treatment resistance, thus, concomitant targeting of multiple pathways might avoid resistance and offer a better clinical outcome.

TMZ is tested in trials alongside other substances such as: morphine, sulforaphane and nimotuzumab.

Low-dose TMZ and morphine reduce tumour growth and chemoresistance, as morphine inhibits P-glycoprotein 1 that are responsible for TMZ resistance.

Another combination that inhibits tumour growth and induce cell death in chemo resistant cell lines is TMZ and sulforaphane (transcriptional NF-kB inhibitor).

TMZ is also being studied in combination with nimotuzumab (monoclonal antibody against epidermal growth factor receptor).

Epidermal growth factor receptor (EGFR) gene is frequently altered in GBM and its amplification enhances tumorigenicity.

Blocking this pathway via nimotuzumab alongside TMZ is currently being studied and displayed so far antitumor effect in GBMs overexpressing EGFRvIII [4,12,13].

Doxorubicin (DOXO) is an anticancer drug most commonly used in the management of breast, bladder, ovarian and thyroid cancer acting by stabilizing topoisomerase II and preventing replication.

As other chemotherapeutics, DOXO presents side effects such as: bone marrow suppression, cardiotoxicity and nephrotoxicity.

In the current study, the effects of combined treatment with temozolomide and doxorubicin are studied in glioblastoma cell line [14].

Materials and Methods

Cell lines and culture conditions

The study was carried out in the GB2B cellular line consisting of glioblastoma cells obtained from patients diagnosed at the "Bagdasar-Arseni" Emergency Hospital, Bucharest, Romania.

Patients provided a written consent.

Cells were cultured in Minimum Essential medium (MEM) with 10% fetal bovine serum (FBS), 2mM glutamine, penicillin (100IU/mL) and streptomycin (100IU/mL) and raised in tissue culture flasks in a humidified incubator at 37°C and 5% carbon dioxide.

The cell lines were established by standard procedures [15].

Cell treatment

Cells were seeded in 96-well plates (0.5-1-3×103 cells/well) and the study was conducted as follows: one control consisting of GBM cells treated with diluents, one group treated with 10μM and 100μM of DOXO, cells treated with 10μM and 100μM of temozolomide and cells treated with a combination of the two drugs in a reduced dose (10μM of doxorubicin and 10μM of temozolomide).

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The cytotoxic effect was assessed at 7, 10 and 14 days.

The chemotherapeutic drugs were added every two days. Every study was performed three or four times.

Liquid handling

The media containing GBM cells, chemotherapeutic agents and liquid reagents was dispensed using an EpMotion 5070 instrument (Eppendorf, Hamburg, Germany) into 96-well culture plates at a density of 1000-3000 cells/well.

Plates were incubated for 24 hours using standard MEM in a humidified medium at 37°C and 5% carbon dioxide.

Cells were washed two times with 100µL medium without serum and received standard medium (200µL), single agent either doxorubicin (10µM or 100µM) or temozolomide (10µM or 100µM) and a combination of doxorubicin and temozolomide (10µM each).

Every study was performed three or four times.

Proliferation assay

The cytotoxic effect of the treatment (single agent administration or dual therapy) on the GBM cell line GB2B was analyzed by MTT assay (Roche Diagnostic GmbH, Basel, Switzerland).

Cells were seeded in 96-well culture plates at a density of 3000 cells/well with 200µL medium in six replicates.

After each treatment, 10µL MTT solution was added to every well and incubated for 4 hours at 37°C until purple formazan crystals were formed.

The metabolically active cells are the only ones able to cleave the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide diphenyl] and form the purple precipitates.

100µL solubilization buffer was used to induce lysis and then the optical density (OD) was measured, using a spectrophotometer.

Statistical analysis

Statistical analysis of the cell viability was performed by using Microsoft Excel Student's t test with one-tailed distribution and the Analysis of variance test (ANOVA) and t-test were used to analyze the variations between study groups, where values with $P<0.05$ were statistically significant.

Drug interactions were categorized as having an additive inhibit inhibitory effect if I1, 2=I1+I2, a synergistic effect if I1,2>I1+I2 and an antagonistic effect is I1,2<I1+I2.

Results were exhibited as mean±standard deviation (SD).

Every study was performed three or four times.

Results

1. Doxorubicin treatment induces cytotoxicity in glioblastoma cells

This part of the study analyses the cytotoxic effect of DOXO on glioblastoma cells using increasing concentrations (10µM and 100µM) of drug.

The viability of the cells was determined on the 7th, 10th and 14th day after drug administration.

During the 14 days course of DOXO, it can be observed that the viability has the tendency to decrease while administering the highest concentration of the drug (100µM and increasing cell time exposure, thus the strongest effect of the drug was observed 14 days after treatment, with a cellular death rate of roughly 53%.

The first 7 days of treatment showed a 34.6% death rate at 10µM DOXO and a 40.9% death rate at 100µM DOXO (Figure 1A).

At the 10th day time mark, cell viability maintained a descending trend while increasing the concentration (cell death rate of 37.8% at 10µM and of 45.6% at 100µM) as seen in Figure 1B.

The highest cytotoxic effect can be observed at 14th day time mark (46% at 10µM and 53% at 100µM), in Figure 1C.

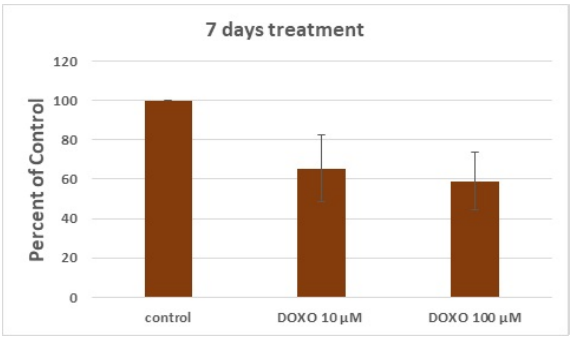

Figure 1A. Effect of doxorubicin on glioblastoma cells (GB2B cell line) viability after 7 days of treatment. Cells were harvested in standard medium and allowed to grow to 70% confluence. Cells were transferred to multi-well plates and were treated with 1µM and 100µM DOXO. The resulting cytotoxic effect on the 10µM group and on the 10µM group is summarized as percent of control and a mean value was obtained after three experiments.

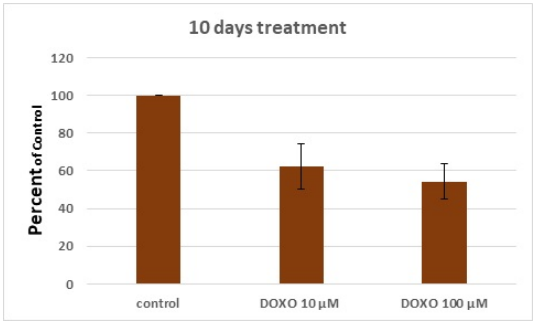

Figure 1B. Effect of doxorubicin on glioblastoma cells (GB2B cell line) viability after 10 days of treatment. Cells were harvested in standard medium and allowed to grow to 70% confluence. Cells were transferred to multi-well plates and were treated with 10µM and 100µM DOXO. The resulting cytotoxic effect on the 10µM group and on the 100µM group is summarized as percent of control and a mean value was obtained after three experiments.

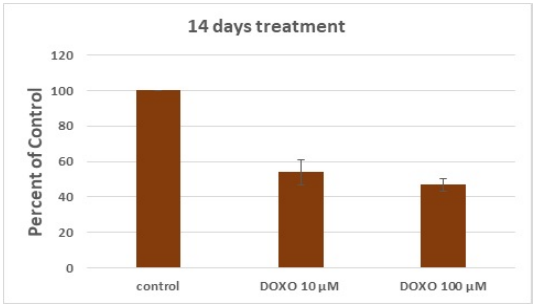

Figure 1C. Effect of doxorubicin on glioblastoma cells (GB2B cell line) viability after 14 days of treatment. Cells were harvested in standard medium and allowed to grow to 70% confluence. Cells were transferred to multi-well plates and were treated with 10µM and 100µM DOXO. The resulting cytotoxic effect on the 10µM group and on the 100µM group is summarized as percent of control and a mean value was obtained after three experiments.

2. Temozolomide treatment induces cytotoxicity in glioblastoma cells

TMZ exposure of GBM cells showed a decreasing viability rate with the progressively higher drug dosage and time exposure.

After 7 days treatment, the proportion of cells that survive was 65.3% at a concentration of 10µM and 59% at 100µM concentration (Figure 2A).

Unexpectedly, cell viability increased with 4% after 10 days of treatment at a concentration of 10µM TMZ, but decreased to 57% at 100µM TMZ in comparison to the 100µM TMZ at 7 days (Figure 2B).

The strongest cytotoxic effect obtained was at 14th day mark, cell viability decreasing to approximately 59% at 10µM and to respectively 45% at 100µM (Figure 2C).

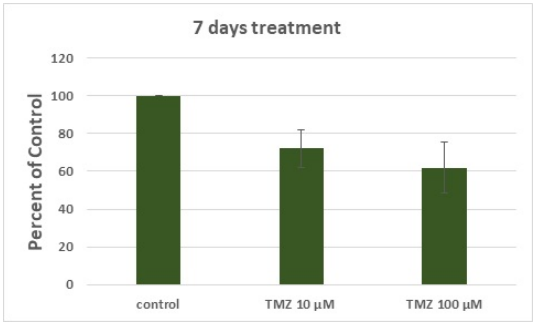

Figure 2A. Effect of temozolomide on glioblastoma cells (GB2B cell line) viability after 7 days of treatment. Cells were harvested in standard medium and allowed to grow to 70% confluence. Cells were transferred to multi-well plates and were treated with 10µM and 100µM TMZ. The resulting cytotoxic effect on the 10µM group and on the 100µM group is summarized as percent of control and a mean value was obtained after three experiments.

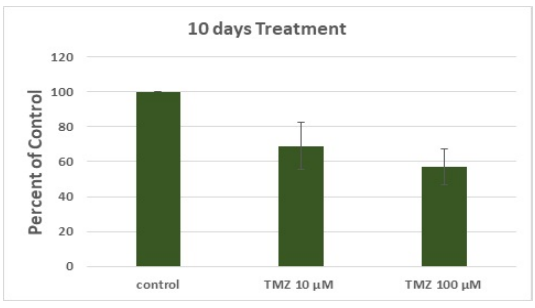

Figure 2B. Effect of temozolomide on glioblastoma cells (GB2B cell line) viability after 10 days of treatment. Cells were harvested in standard medium and allowed to grow to 70% confluence. Cells were transferred to multi-well plates and were treated with 10µM and 100µM TMZ. The resulting cytotoxic effect on the 10µM group and on the 100µM group is summarized as percent of control and a mean value was obtained after three experiments.

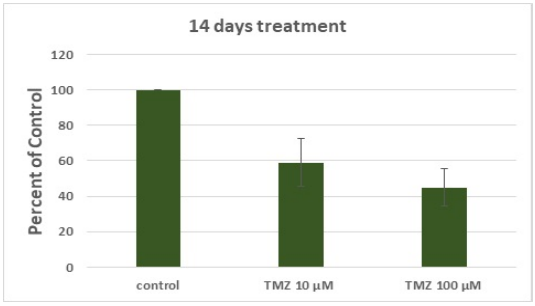

Figure 2C. Effect of temozolomide on glioblastoma cells (GB2B cell line) viability after 14 days of treatment. Cells were harvested in standard medium and allowed to grow to 70% confluence. Cells were transferred to multi-well plates and were treated with 10µM and 100µM TMZ. The resulting cytotoxic effect on the 10µM group and on the 100µM group is summarized as percent of control and a mean value was obtained after three experiments.

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3. Combined treatment with temozolomide and doxorubicin did not exert a synergistic cytotoxic effect on glioblastoma cells

The combined cytotoxic ability of both drugs was analyzed in comparison to the single administration.

At 7 days post-administration, cell viability was approximately 64% for the dual administration group (TMZ 10µM+DOXO 10µM), while cell viability for the single administration groups was 65.3% for DOXO 10µM and, respectively, 72% for TMZ 10µM, resulting in a similar cytotoxic effect for the dual combination group and for the doxorubicin group (Figure 3A).

At 10 days mark, the cytotoxic effect was 30.9% for TMZ 10µM, 37.8% for DOXO 10µM and 40.7% for TMZ 10µM+DOXO 10µM (Figure 3B).

The maximum death rate was observed at 14 days, but without exerting synergism. TMZ 10µM induced cellular death in 41% of the GBM cells, DOXO 10µM displayed a 46% cell death and the combined group induced cell death in half of the analyzed cells (Figure 3C).

It can be observed that throughout the assessed time frames, all of the groups displayed a continuously decreasing cell population, but the difference between the dual therapy and the DOXO group was of only 2-4% and between the dual therapy and the TMZ group was of 8-10%.

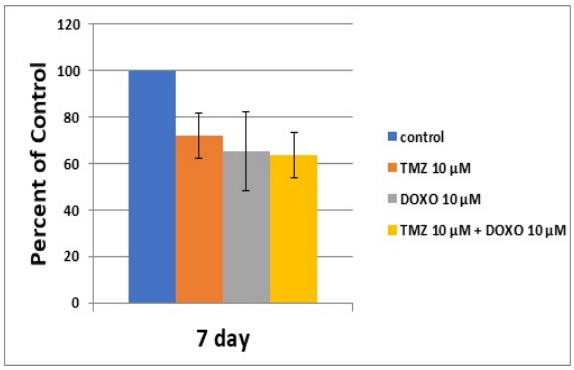

Figure 3A. Cytotoxic effect of combined treatment on glioblastoma cells (GB2B cell line) after 7 days of treatment. Cells were harvested in standard medium and allowed to grow to 70% confluence. Cells were transferred to multi-well plates and were treated with 10µM DOXO, 10µM TMZ and the combination of the two chemotherapeutic drugs in low doses (10µM TMZ+10µM DOXO). The resulting cytotoxic effects on the three groups after 7 days are summarized as percent of control and a mean value was obtained after three experiments.

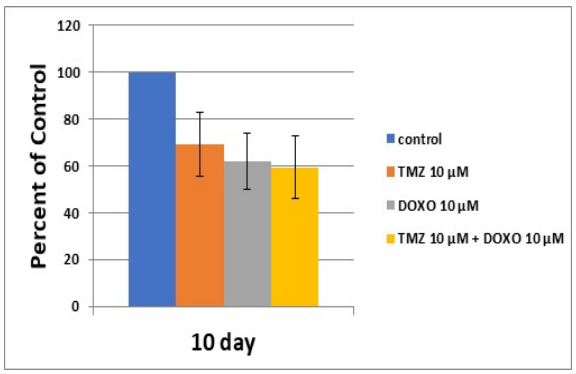

Figure 3B. Cytotoxic effect of combined treatment on glioblastoma cells (GB2B cell line) after 7 days of treatment. Cells were harvested in standard medium and allowed to grow to 70% confluence. Cells were transferred to multi-well plates and were treated with 10µM DOXO, 10µM TMZ and the combination of the two chemotherapeutic drugs in low doses (10µM TMZ+10µM DOXO). The resulting cytotoxic effects on the three groups after 10 days are summarized as percent of control and a mean value was obtained after three experiments.

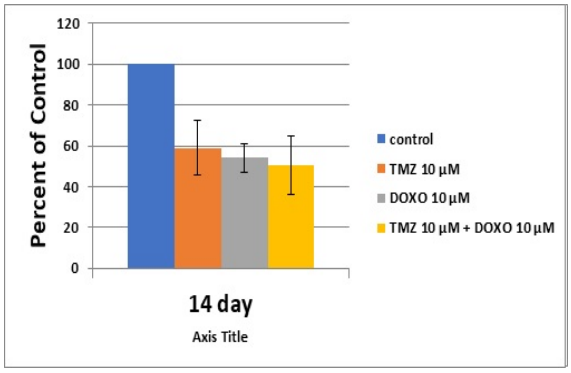

Figure 3C. Cytotoxic effect of combined treatment on glioblastoma cells (GB2B cell line) after 7 days of treatment. Cells were harvested in standard medium and allowed to grow to 70% confluence. Cells were transferred to multi-well plates and were treated with 10µM DOXO, 10µM TMZ and the combination of the two chemotherapeutic drugs in low doses (10µM TMZ+10µM DOXO). The resulting cytotoxic effects on the three groups after 14 days are summarized as percent of control and a mean value was obtained after three experiments.

To compare the efficacy of the combined treatment with the monotherapy, the interaction between TMZ and DOXO concomitant treatment was calculated.

As seen in the Table 1, in the GB2B cells, none of the combinations used in this study proved to be synergic or additive.

Table 1. The interaction between combined treatment in GB2B cells.

Time (days)	TMZ (μM)	DOXO (μM)	Predicted survival	Observed survival	Effect
7	10	10	0.47	0.64	SUB
10	10	10	0.43	0.59	SUB
14	10	10	0.32	0.5	SUB

Discussions

The aggressiveness of GBM and failure to provide an efficient treatment makes GBM one of the most dreaded cancers.

Monotherapy has been proven to be less efficient than combination therapy, thus one of the promising approaches to treating this aggressive tumour is combination therapy and many chemotherapeutic agents are currently under investigation in dual therapy [4].

TMZ represents the current approved therapy for GBM, being used for almost two decades in combination with radiotherapy and surgery.

Although it contributed to a better median survival rate, its efficiency is limited by tumour resistance and recurrence [9].

Since its clinical effect is mostly palliative, many in *vitro* studies are engaged into finding new efficient drug combinations. One such study showed a synergistic cytotoxic effect of micellarized cyclopamine (MCyp) and TMZ [16].

Other *in vitro* and *in vivo* studies assessing the combined effect of TMZ and different pharmacological agents proved a reduction in tumour growth (TMZ and morphine) and an increased chemosensitivity (TMZ and sulforaphane) [17,18].

DOXO is another very effective chemotherapeutic agent employed in a variety of cancers and with a different mechanism of action than TMZ.

O Maksimenko et al. have studied in an *in vivo* experiment the anti-tumour effect of optimized DOXO loaded in poly (lactide-co-glycolide) nanoparticles which was subsequently confirmed [19].

In our previous study, we showed that the low-passage GB8B cell line was sensitive to both DOXO and TMZ, exhibiting apoptosis in 66.5% of cells when treated with 100μM TMZ at 14 days and showed a similar effect when treated with higher doses of DOXO at 14 days post administration.

Nonetheless, the combination therapy did not exhibit a synergistic effect [20].

This study was undertaken to assess the effect of combination between TMZ and DOXO in another GMB cell line, GB2B.

Here, we also find that in monotherapy, each drug produced a cytotoxic effect (maximum cytotoxic effect of 53% for 100μM DOXO at 14 days and 55% for 100μM TMZ at 14 days), but in dual therapy, they failed to exert a synergistic effect.

Conclusions

This study investigated the cytotoxic effect of DOXO in combination with TMZ in GBM cells *in vitro*.

Our results showed that both DOXO and TMZ induce cytotoxicity in GBM cells in single administration, however, the combined treatment failed to act synergistically in inducing cell death in GBM cells.

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Carina Baloi, Alexandru Oprita and Liliana Eleonora Semenescu contributed equally to this work and thus share equal contributions.

Conflict of interests

None to declare.

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