Biomedicine & Pharmacotherapy 94 (2017) 21–26

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Combined calcitriol and menadione reduces experimental murine triple negative breast tumor

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ARTICLE INFO

Article history:
Received 17 May 2017
Received in revised form 7 July 2017
Accepted 11 July 2017

Keywords:
Calcitriol
Menadione
Triple negative breast cancer
In vivo tumor growth
Cell death

ABSTRACT

Background: Calcitriol (D) or 1,25$(OH)_2D_3$ inhibits the growth of several tumor cells including breast cancer cells, by activating cell death pathways. Menadione (MEN), a glutathione-depleting compound, may be used to potentiate the antiproliferative actions of D on cancer cells. We have previously shown *in vitro* that MEN improved D-induced growth arrest on breast cancer cell lines, inducing oxidative stress and DNA damage *via* ROS generation. Treatment with MEN + D resulted more effective than D or MEN alone.
Objective: : To study the *in vivo* effect of calcitriol, MEN or their combination on the development of murine transplantable triple negative breast tumor M-406 in its syngeneic host.
Methods: Tumor M-406 was inoculated s.c., and when tumors reached the desired size, animals were randomly assigned to one of four groups receiving daily i.p. injections of either sterile saline solution (controls, C), MEN, D, or both (MEN + D). Body weight and tumor volume were recorded three times a week. Serum calcium was determined before and at the end of the treatment, at which time tumor samples were obtained for histological examination.
Results: None of the drugs, alone or in combination, affected mice body weight in the period studied. The combined treatment reduced tumor growth rate (C *vs.* MEN + D, $P < 0.05$) and the corresponding histological sections exhibited small remaining areas of viable tumor only in the periphery. A concomitant DNA fragmentation was observed in all treated groups and MEN potentiated the calcitriol effect on tumor growth.
Conclusions: As previously observed *in vitro*, treatment with MEN and D delayed tumor growth *in vivo* more efficiently than the individual drugs, with evident signals of apoptosis induction. Our results propose an alternative protocol to treat triple negative breast cancer, using GSH depleting drugs together with calcitriol, which would allow lower doses of the steroid to maintain the antitumor effect while diminishing its adverse pharmacological effects.

1. Introduction

Although calcitriol (D) or 1,25-dihydroxyvitamin D_3 is well known for its effects on calcium and phosphate metabolism, antiproliferative non-classical effects were recently described for this steroid hormone [1–4]. Moreover, calcitriol has been shown to regulate almost all cancer hallmarks such as cellular proliferation, differentiation, cell death, angiogenesis, metastasis, immunomodulation and inflammation [5,6]. On the other hand, the antitumor activity of menadione (MEN) or vitamin K_3, a chemically synthesized compound that appears to have anticancer effects [7,8], is still not completely understood. However, MEN is a glutathione (GSH)-depleting drug and tumor resistance is associated with high intracellular GSH levels. MEN or D,L-buthionine-S, R-sulfoximine (BSO), another GSH-depleting drug, are used in clinical practice and may act as sensitizers when administered in combination with conventional chemotherapeutic agents in various cancer types [9,10].

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http://dx.doi.org/10.1016/j.biopha.2017.07.058
0753-3322/

Breast cancer (BC) cells have been shown to be targets of vitamin D compounds [4,11]. We have previously demonstrated that D in combination with MEN or BSO, elicited antiproliferative effects on MCF-7 and other breast cancer cell lines [1,2], and that the inhibitory action on cell growth *in vitro* was even more potent than using D or MEN alone. Moreover, we have shown a similar antineoplastic activity of this combined therapy on colon adenocarcinoma cells [12]. In these studies, the effects of MEN and D on tumor cell growth were associated with oxidative stress induction, cell cycle arrest and activation of cell death pathways.

BCs are often classified by the expression profile of three membrane receptor proteins: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). During the last 15 years, five intrinsic molecular subtypes of breast cancer have been identified through gene-expression profiling, (Luminal A, Luminal B, HER2-enriched, Basal-like, and Claudin-low), which have improved our knowledge of breast cancer biology [13]. Particularly, mRNA profiles have been used to define the prognostic value of these subtypes of breast cancer [14]. The presence or absence of these receptors confers distinctive biological characteristics to these BC subtypes that determine the response patterns to different therapeutic modalities and their clinical outcome. The triple-negative breast cancer (TNBC), a subtype that lacks the expression of ER, PR, and HER2, represents a very heterogeneous group of breast diseases, associated with a significantly higher probability of relapse and poorer overall survival in the first few years after diagnosis [15,16]. One of the BC cell lines studied in our previous work, the human triple-negative breast cancer (TNBC) HMLER cell line [17], was susceptible to calcitriol or GSH-depleting drugs and much more to the combined treatment. In the conditions of the experiment, the HMLER cells treated with GSH-depleting drugs, D or both showed a significant delay in their doubling time compared to the control [2]. Since cytotoxic chemotherapy is currently the only treatment option for TNBC [18], the aim of the present work was to analyze the effect of calcitriol, MEN or their combination in an *in vivo* setting, using the CBi-IGE murine model of a transplantable triple-negative breast tumor [19].

2. Materials and methods

2.1. Chemicals

MEN was obtained from Sigma (St. Louis, MO, USA) and was used at 2 mg/kg body weight (BW). $1,25(OH)_2D_3$ was a generous gift from Roche Diagnostic GmbH (Mannheim, Germany), and used at 0.15 μg/kg of BW. All other reagents were of analytical grade.

2.2. Experimental design

2.2.1. Animals

Twenty-seven CBi female mice (11–14 weeks old) of the CBi-IGE colony from the Animal Facilities of the Instituto de Genética Experimental, Facultad de Ciencias Médicas, Universidad Nacional de Rosario (from here on, CBi-IGE stock), were used. The CBi-IGE stock comprises five genetically distinct lines obtained from selection experiments, currently in their 140th generation of selective breeding. A description of the selection procedure is given elsewhere [20]. The average weight of the animals at the beginning of the experiment was 32 g. Mice were kept in the IGE animal facility, in a room with a constant temperature of $24 \pm 2\,^{\circ}C$, a relative humidity of $50 \pm 10\%$ and a 12-h-on/12-h-off light cycle. Animals were fed the same diet (Gepsa Feeds pelletized, Grupo Pilar S.A., Córdoba, Argentina) and water *ad libitum*. Experiments were performed during the first half of light cycle. Mice were treated following the institutional regulations (Facultad de Ciencias Médicas, Universidad Nacional de Rosario, permit # 081/2014) which comply with the guidelines issued by the Institute for Laboratory Animal Resources, National Research Council, USA (2011) and the Canadian Council on Animal Care (1998). All experiments were performed with prior approval from the Bioethics Committee of the Facultad de Ciencias Médicas, Universidad Nacional de Rosario.

2.2.2. Tumor

M-406 is a type B semi-differentiated mammary adenocarcinoma [21,22] that arose spontaneously in an inbred CBi female mouse in 1998 and is maintained *in vivo* in syngeneic mice by serial intraperitoneal implantation every 14 days. When the tumor is inoculated subcutaneously (*s.c.*) in the line of origin, the lethality is 100% (100% take and 0% regression). M-406 was characterized as a triple negative tumor (ER-, PR-, HER2-) [19].

2.2.3. Experimental approach

Mice were challenged *s.c.* with M-406 by trocar in the right lateral flank. When tumors reached an approximate size of 150 mm^3 (5 days post-inoculum), the animals were randomly divided into four experimental groups which were treated daily, from Monday to Friday, with sterile *i.p.* injections of saline (Control, Group C, n = 6), 2 mg MEN/kg BW (Group MEN, n = 7), 0.15 μg 1,25 $(OH)_2D_3$/kg of BW (Group D, n = 7) or the combination of both drugs (Group MEN + D, n = 7). Body weight (g) and minor/major tumor diameters (mm) were measured, and the general health status monitored, three times a week throughout the experiment. Tumor volume (TV, mm^3) was estimated according to the formula TV = (minor diameter)^2 x major diameter x 0.4 [23,24]. Tumor volume was plotted as a function of time (days), and the graph was fitted with an exponential function. A parameter of this curve, the tumor doubling time (Tdt), is clinically relevant [25], and was used to compare the effect of the different treatments. Tumor growth rate was also calculated as the ratio between volumes measured at two consecutive times (rate of tumor volume increase, as depicted in Fig. 1). Mice were sacrificed by cervical dislocation when tumors from the control group had reached the maximum size permitted by ethical standards. At that time, the tumors were removed and processed for histological examination.

2.3. Histology

Tumors were removed after euthanasia, fixed in buffered 10% formalin solution for 24 h and embedded in paraffin. Five-micron sections were stained with hematoxylin and eosin (H&E) for histopathological studies and for counting nuclei in mitotic cell division.

2.4. TUNEL technique

DNA fragmentation was detected by the terminal transferase mediated dUTP nick-end labeling assay (TUNEL) employing ApopTag Plus Peroxidase *in situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA), as previously described [26]. The detection of peroxidase activity was done using 3,3 – diaminobenzidine (DAB; Thermo Scientific, Rockford, USA), as a chromogen and the sections were counterstained with 0.5% (w/v) methyl green for 10 min at room temperature. The apoptotic cells were counted at 400× magnification in at least three sections from three animals for each treatment, which was accomplished by two independent researchers in a blinded fashion. Positive and negative controls were also performed. Positive controls were established using the slides contained in the same kit following the manufacturer's instructions. Sections processed without TdT

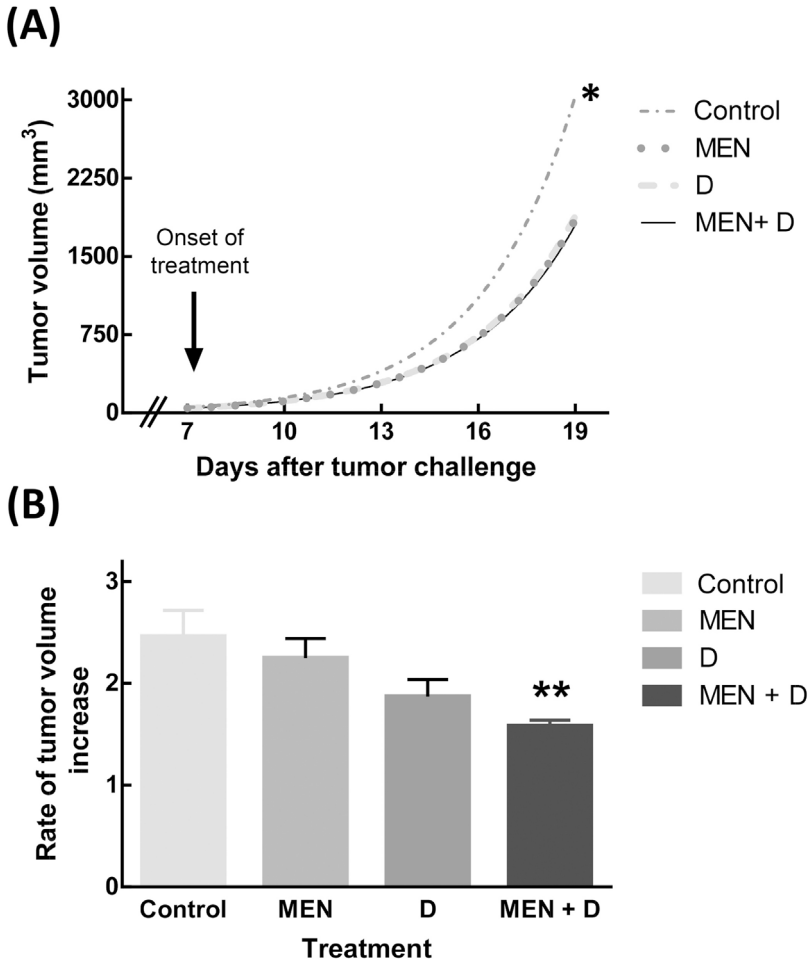

Fig. 1. Effects of menadione (MEN), 1,25$(OH)_2D_3$ (D), or both drugs (MEN + D) on tumor growth in CBi-IGE mice challenged with M-406.
(A) Tumor growth curves of the different treatment groups, fitted with an exponential growth model, showing a significant delay in tumor progression when compared with the Control group ($P = 0.0030$). * significantly different from the treated groups. **(B)** Tumor volume rate between days 17 and 19 after challenge. Mean and SEM of each group are plotted. Differences among groups were analyzed with a one-way ANOVA ($P = 0.0109$), followed by Bonferroni's multiple comparisons test. ** significantly different from the Control group.

enzyme in the labeling reaction mix were used as negative controls.

2.5. Blood extraction and processing

Blood samples were obtained from the mouse tail vein in two experimental stages: 2 days before tumor challenge and immediately previous to euthanasia. Blood was collected and centrifuged 10 min at 12000 rpm. Serum was separated and kept at −20 °C until use. Serum calcium was measured spectrophotometrically using CaColor AA (Wiener Lab, Rosario, Argentina), according to the manufacturer's instructions. Serum calcium was considered normal between 8.8 and 10.4 mg/dL [27].

2.6. Statistical analysis

The statistical significance of the difference among treatment groups was examined with a one-way analysis of variance (ANOVA), followed by theBonferroni post-test to compare pairs of groups, or the Kruskal-Wallis non-parametric test and Dunn's multiple comparisons test, when appropriate [28]. Differences were considered significant if $P < 0.05$. Analyses were carried out with the SPSS software (version 22.0) for Windows 8.1 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Calcitriol and MEN effects on tumor volume and serum calcium

According to the tumor growth curves, fitted with an exponential growth model, calcitriol, MEN and the combined treatment induced a significant delay in tumor progression ($P = 0.003$) in comparison with the control group (Fig. 1A). Tumor growth rate, calculated as the ratio between volumes measured on days 17 and 19 after challenge, was used as another parameter to evaluate the effect of these drugs. Mice treated with the combination of D and MEN showed a smaller tumor growth rate compared to the controls ($P \leq 0.010$) demonstrating that the combined therapy was able to delay triple negative M-406 growth more efficiently than the single drug exposure (Fig. 1B). These effects were observed without any change in the body weight of the experimental animals (Mean ± SEM, C: 34.8 ± 1.0 g, MEN: 33.4 ± 2.0 g, D: 34.1 ± 2.0 g, MEN + D: 34.8 ± 2.0 g, at the end of the treatments, $P = 0.334$). At the beginning of the experiment mean serum calcium in CBi mice was 10.14 ± 0.47 mg/dL. Treatment with D, given singly or in combination with MEN, induced a mild increase in serum calcium at the end of the experiment (Mean ± SEM, MEN: 11.82 ± 0.93 mg/dL; D: 13.87 ± 0.58 mg/dL; MEN + D: 14.2 ± 0.7 mg/dL, $P = 0.001$).

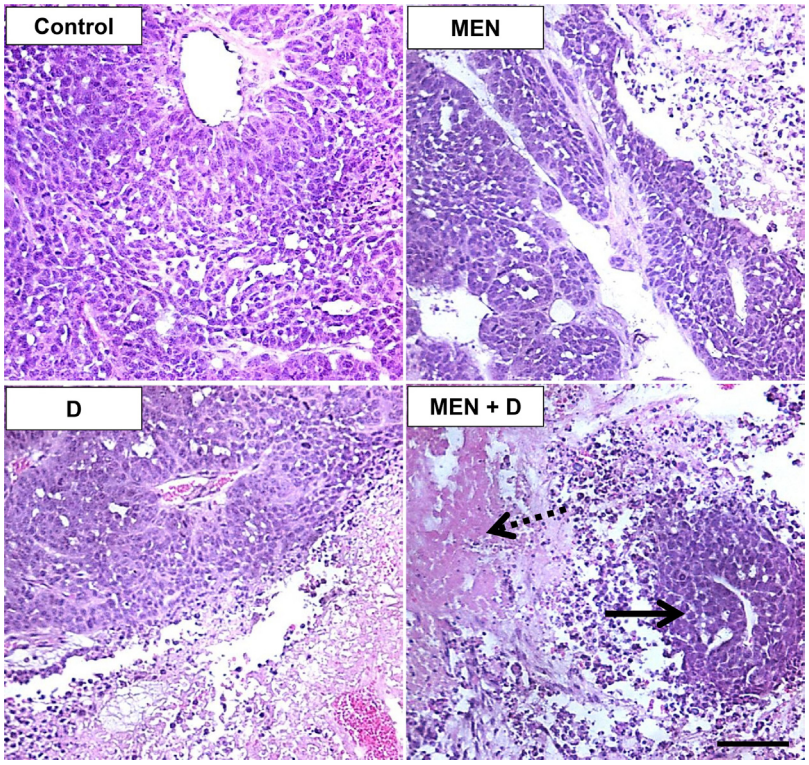

Fig. 2. Representative micrographs illustrating M-406 histology.
Tumor tissue samples obtained 19 days after challenge show angiocentric distribution areas and abundant mitosis (Control, MEN and D). Treatment with both Menadione and Calcitriol (MEN + D) show tumor remains of perivascular growth on necrotic areas (broken line arrow) and inflammatory infiltrates (arrow). H&E staining, bar = 20 μm.

3.2. Tumor histology

Tumor histological analysis from the different experimental groups showed similar and distinctive characteristics (Fig. 2). All histological sections showed proliferation of neoplastic epithelial cells with comparable characteristics as well-defined nests, cords, and glandular/tubular differentiation. Most of the cells were arranged in an angiocentric growth pattern. Stromal invasion and expansive edges were observed. The stromal tissue presented rich vascularity, and mixed inflammatory infiltrate. Many mitotic figures were detected, and areas of tumor necrosis occurred in all cases. In contrast, tumor tissue samples from the groups receiving calcitriol and, particularly in the combined treatment, showed areas of ischemic necrosis that exceeded the conserved ones, and were mainly found in the periphery of the tumor.

3.3. Mitosis counting and DNA fragmentation analysis

None of the treatments modified cell proliferation, since the number of mitotic nuclei resulted similar in all experimental groups (Control: 1.22 ± 0.4; MEN: 1.58 ± 0.37; D: 1.42 ± 0.27; MEN + D: 1.50 ± 0.34, $P = 0.892$). In contrast, D, MEN, or their combination induced a significant DNA fragmentation (Fig. 3). Moreover, MEN potentiated the effect of D on tumor DNA fragmentation. Altogether, these observations suggest that a programmed cell death pathway may be activated in this model.

4. Discussion

Breast cancer is the most common tumor and the leading cause of cancer-related deaths among the female population worldwide

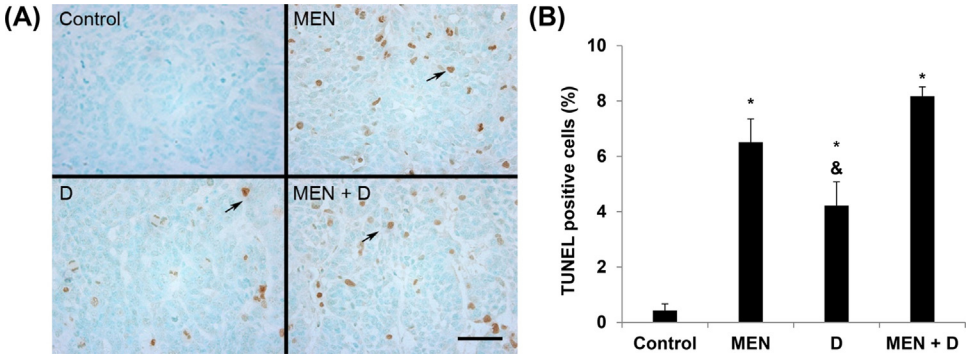

Fig. 3. Nuclear DNA fragmentation in CBi-IGE mice challenged with M-406 and treated with MEN, D or MEN + D.
Tissue samples were obtained on day 19 after challenge. **(A)** The images show tumor cells after TUNEL staining. The brown color indicates positivity (arrows), bar = 20 μm. **(B)** Mean ± SEM of TUNEL positive cells percentage for each experimental group. Differences among groups were analyzed with ANOVA and Bonferroni; $^{*}P \leq 0.01$ *vs* Control, $^{\&}P \leq 0.01$ *vs* MEN and MEN + D.

[29]. Standard chemotherapies are often based on agents that target ER, PR or the expression of the HER2 receptor. However, TNBCs, which are detected in almost 15% of breast cancer patients, have fewer therapeutic options. The lack of identified molecular targets in the majority of TNBCs implies that chemotherapy remains the treatment of choice for patients with TNBC [15]. Moreover, TNBC is associated with poor prognosis and lack of sustained response to conventional chemotherapeutic agents [30]. TNBCs are also considered the breast cancer subtype more poorly differentiated, so a better understanding of how to treat them is needed [31].

Calcitriol plays an essential role in the normal development of the mammary gland, and the progression of breast cancer and breast epithelial cell transformation is associated with deregulation of the vitamin D pathway [32,33]. On the other hand, MEN proved to reduce tumor growth in different *in vitro* and *in vivo* mammary cancer models including TNBC [10]. We have previously demonstrated that the antiproliferative effect of calcitriol on MCF-7 cells, an *in vitro* ER-positive breast cancer model, is enhanced by MEN [1] or BSO [2], both GSH depleting drugs. The antitumor action was the result of the oxidative stress triggered by this treatment, which finally led to cell apoptosis. Interestingly, HMLER cells, which are considered basal TNBC cells [34], resulted very sensitive to BSO and much more to the combined treatment, although less susceptible to D than MCF-7 cells [2]. These results suggest that the intensity of calcitriol response may depend on the cancer cell type.

Due to the promising results obtained in the *in vitro* assays, we decided to use the same treatment approach in an *in vivo* setting. Combining calcitriol and MEN was more efficient to delay tumor growth *in vivo* in mice bearing the triple negative syngeneic M-406 breast cancer. Tumor growth rate was significantly reduced, and the antitumoral effects resulted more potent than those with D or MEN used alone. The observed antitumoral activity did not affect mice body weight in the period analyzed. Furthermore, the dose of calcitriol used throughout this study produced a mild increment in serum calcium without typical symptoms of hypercalcemia such as conjunctivitis, chills, thirst, lethargy or any physical changes [35]. Interestingly, when tumor histology was analyzed, ischemic necrosis was observed in the three treated groups. However, the group receiving the combined treatment presented extensive necrosis, predominating in the center of the tumor and exceeding the conserved areas, with only small remnants of tumor preserved in the periphery, showing an angiocentric distribution. Individual and combined treatment induced DNA fragmentation, thus suggesting that the induction of apoptosis is part of the molecular mechanism of action of D or MEN, as we have previously reported in our *in vitro* approach [1,2]. However, MEN enhanced calcitriol apoptotic effects when administered together. Therefore, it should be possible to increase the efficacy of the anticancer effect of calcitriol by using MEN concomitantly, which would make breast cancer cells more susceptible to apoptosis due to changes in their redox status.

As mentioned above, there are many reports in the literature showing the beneficial effects of calcitriol and its analogs against different breast cancer models ([36],and references therein). However, GSH-depleting drugs (MEN or BSO) are less common as therapeutic options for this pathology [10]. The novelty of the present work is that the combination of MEN with calcitriol might result in an alternative to treat either breast cancers resistant to the standard protocols or TNBCs.

5. Conclusion

This study shows that co-administration of calcitriol and GSH-depleting drugs may be an option to treat triple-negative breast tumors since their combination provoked a reduction in the growth rate of a transplantable murine TNBC as well as an associated increment in tumor cell death. As TNBCs subtypes are one of the least responsive breast cancers to available targeted therapies, the combination of D and MEN is an alternative that deserves to be further investigated to improve the therapeutic outcome.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Funding

None of the funding institutions was involved in the study design, collection, analysis and interpretation of data, writing of the report, and decision to submit the article for publication.

Acknowledgements

LB, GP, VR, and NTT are members of the Investigator Career from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas). SG is the recipient of a CONICET fellowship. This work was supported by grants from CONICET and SECYT (UNC), Argentina. LH is a member of the Investigator Career from CIUNR (Consejo de Investigaciones, Universidad Nacional de Rosario). LH and VR are grateful to SECTeI (FCM-UNR) for subsidizing work done at the Instituto de Genética Experimental. The authors wish to especially thank Roche Diagnostics GmbH (Mannheim, Germany) who provided us with calcitriol.

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