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Investigation of In-Vitro Biological Behavior and Pro-Angiogenic Potential of Baicalein as Hypoxia-Mimicking Agent

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Abstract

In bone tissue engineering, vascularization is important for cell survival and a successful surgical procedure. Vascularization can be enhanced by hypoxia, but it is not possible to create hypoxia in the body. The present study describes functional biological potential of baicalein, a chemical hypoxia-mimicking agent and hypoxia-enhanced in vitro pro-angiogenesis of human osteosarcoma cell line. Relationship between expression of hypoxia-inducing factor-1a (HIF-1α) and pro-angiogenesis in oteosarcoma cell line without affecting osteogenic potential were studied. The HIF-1α pathway along with VEGF expression were used to test for pro-angiogenic effects, while ALP expression was used to measure any changes in osteoinductive potential. Baicalein ($C_{15}H_{10}O_5$) showed increased HIF-1α stabilization and nuclear translocation, hypoxia responsive element activation and VEGF expression, all of which point to a marked pro-angiogenic effect. ALP expression reduced significantly in response to baicalein when compared to osteogenic medium. These studies demonstrated that baicalein ($C_{15}H_{10}O_5$) has a high potential as a viable hypoxia mimicking agent.

Keywords: Hypoxia, angiogenesis, osteosarcoma, VEGF, HIF-1α, baicalein

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Introduction

Bone is the main supporting system in the human body. Surgical intervention is often a necessity in the treatment of various types of bone defects, and the regeneration of bone tissue remains a major challenge in these surgical procedures. Although bone grafting has been shown to be a highly successful therapy (Salgado et al., 2004) the use of autologous bone and allogeneic bone is still far from optimal. When using autologous bone grafts, 10 to 30 percent of patients develop complications due to the additional surgery required to harvest healthy bone tissue (Arrington, E.D., 1996). This is not a problem with the use of allogeneic bone, but the quality of allogeneic grafts is typically lower than that of autologous grafts (Damien and Parsons, 1991). To overcome the drawbacks of current grafts, bone tissue engineering (BTE) using bone marrow stem cells has been suggested as a promising technique for reconstructing bone defects. Until recently no convincing results of the use of this technique in humans have been reported (Gaalen, 2008, Meijer et al., 2007).

Cell survival is the most important requirement for achieving clinical success in cell-based bone tissue engineering (Meijer et al., 2007). A critical obstacle in tissue engineering is inability to maintain large masses of living cells viable upon transfer from the *in vitro* culture conditions into the host *in-vivo* (Ko et al., 2007). *In-vivo*, most cells located more than a few hundred micrometers away from capillaries do not survive, because transportation of soluble substances by diffusion alone is insufficient at locations far away from the capillaries. Metabolically active cells must reside within a range of 150-200 μm of a capillary, which correlates to the diffusion limit of oxygen (Novosel et al., 2011, Phelps et al., 2010). For this reason, tissue engineering techniques have mainly been applied on avascular tissues or tissues that can do without a vascular supply (Frerich et al., 2001).

Current research is tackling the problem of vascularization with four distinct strategies, all of which have demonstrated moderate success in animal models (Phelps et al., 2010). The first strategy is based on the inclusion of growth factors such as vascular endothelial growth factor (VEGF) and bFGF into scaffolds. The release and diffusion of these cytokines can induce vessels in surrounding tissues to grow into the

scaffold (Ko et al., 2007). The second strategy relies on the delivery of progenitor cells alone or in combination with growth factors and biomaterial scaffolds. Through transplantation of mixtures of endothelial and mesenchymal progenitor cells encapsulated in biological extracellular matrix (ECM), tissue engineering research has progressed to the point of predictably and repeatedly producing stable vasculature in a variety of animal models (Koike et al., 2004). The third strategy is based on the delivery of vascular-inductive engineered materials. Major effort has been put into the development of fully synthetic or well defined biological matrices with potent pro-angiogenic properties. These properties can be manifested through encapsulation of co-culture systems of endothelial and mesenchymal stem cells or through cell-free smart materials directly recruiting vascular in growth (Phelps et al., 2010, Kraehenbuehl, 2008).

A promising method of enhancing vascularization in engineered tissues is to promote the secretion of growth factors by cells within the grafts. Exposure to hypoxia has been shown to enhance the differentiation of mesenchymal stem cells into endothelial-like cells that produce angiogenic growth factors. Hypoxia influences the HIF-1 pathway that can activate an elaborate pro-vascularization signaling cascade.

The HIF-1 complex contains two subunits, HIF-1α and HIF-1β, which can dimerize under hypoxic conditions due the attenuated activity of prolyl hydroxylase domain (PHD) containing proteins. The stabilized HIF-1 complex is translocated into the nucleus where it binds to hypoxia responsive elements (HREs) and activates the expression of target genes implicated in angiogenesis, cell growth and survival. More than 70 hypoxia target genes have been identified, including VEGF. Hypoxia-inducible factor (HIF)-1α is a short-lived protein and is ubiquitinated and degraded through the von Hippel-Lindau protein (pVHL)-E3 ubiquitin ligase pathway at normoxia. The HIF-1 pathway has been described in more detail (Xia et al., 2009). The translocation of HIF-1 into the nucleus is not enhanced by hypoxia; instead, the level of oxygen directly regulates the half-life of HIF-1α. This depends not only on the above mentioned PHDs, but also on interaction with von Hippel-Lindau (VHL) tumor suppressor and VHL protein-interacting deubiquitinating enzyme 2, VDU2. Carciac myocytes also appear to be involved in regulating the degradation of HIF-1α. (Semenza, 2010).

A major drawback in using hypoxia to stimulate vascularization is that the lack of oxygen is detrimental to cells, and a careful balance has to be struck between cell survival and promotion of angiogenesis. To overcome this problem a lot of research effort has gone into the search for hypoxia mimics (Xia et al., 2009, Weng et al., 2010, Torii et al., 2011). These are substances that affect the HIF-1 pathway in ways similar to hypoxia exposure, but ideally without compromising cell viability.

A number of compounds have been confirmed to act as hypoxia mimicking agents, including o-phenanthroline, iodochlorhydroxyguinoline, cobalt sulfate heptahydrate ($CoSO_4$), deferoxamine (DFO) and Cobalt chloride ($CoCl_2$) (Xia et al., 2009, Weng et al., 2010, Torii et al., 2011). Furthermore, it has been found that 7-diethylamoni-4-methylcoumarin and 7,12-dimethylbenz(a) anthracence interact with HIF-1α in a different manner from hypoxia. For these compounds both the HREs and secretion of VEGF have been examined, but their effects were less pronounced than those of hypoxia itself (Xia et al., 2009). Certain chemical hypoxia-mimicking agents, such as $CoCl_2$, may result in heavy metal toxicity or inflict security threats to cell microenvironment.

Because of HIF-1-specific prolyl-4 hydroxylases (PHDs) prime degradation of HIF-1, we have endeavored to find a small molecule with the potential of up-regulating the HIF-1 pathway by inhibiting prolyl hydroxylation. Through an in vitro molecular study, we have tested the potential inhibitory effect of baicalein ($C_{15}H_{10}O_5$) on prolyl-4 hydroxylases (PHDs) to increase the HIF-1 content, leading to stimulation of HIF-1-mediated reporter gene action and target gene transcription in tissue culture cells. We chose baicalein ($C_{15}H_{10}O_5$), which is a major component of the dried root of *Scutellaria baicalensis*, widely used in traditional Chinese medical applications (Cho, 2008).

Materials and methods

Cell Culture and Reagents: Human osteosarcoma (MG-63) cell line (Xpand Biotech, Netherlands) was maintained in basic medium: α-modified Eagle's medium (αMEM, Gibco) with 10% Fetal Bovine Serum (FBS, Lonza), 2 mM L-Glutamine (Gibco), 0.2 mM Asorbic Acid (Sigma), 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). For exposure to baicalein a final concentration of 200µM was used. The physiological conditions for cell incubation were 20% O_2, 37°C, but hypoxia conditions were 3-4% O_2 at 37°C. For Alkaline Phosphatase Assay, MG-63 cell line was maintained for 24hr in osteogenic medium containing 100 nmol/L dexamethasone, 10 mmol/L beta-glycerophosphate, and 0.05 mmol/L L-ascorbic acid-2-phosphate. All chemicals used for this investigation were bought from Invitrogen and Sigma and were used according to the manufacturer protocols.

Cell Staining: MG-63 cell lines were seeded at a concentration of 8000 cells/cm^2.To stain the cells, cells were fixed in 4% paraformaldehyde and permeabilized with 0,1% Triton X-100. Triton was blocked with 10% FBS. HIF-1α was stained with 1:50 primary (100 µl, mouse anti human HIF-1α, Abcam) and 1:100 secondary antibody (100 µl, IgG goat anti mouse, Alexa 488, Invitrogen). F-actin was stained with 1:40

Phalloidin 568 and nuclei were stained with 1:100 DAPI. Cells were photographed using the BD Pathway 435 automated confocal fluorescence microscope.

Transfection assay: MG-63 cell lines were seeded at a concentration of 10,000 cells/cm^2. The cells were washed with PBS and 1.9 ml OPTIMEM and 98 µl FUGENE-DNA complex (4% FUGENE, 96% OPTIMEM, 2% DNA (1.277 µg/µl), Roche) was added. After 8 hours the cells were washed with basic medium and medium was refreshed. After confluence baicalein was added and cell lysate was obtained using Lysis reagent. DNA amounts were measured using CyQuant DNA quantification kit per manufacturer instructions. A DNA standard curve was prepared in duplicate, with DNA diluted in DNA lysis buffer. A white 96 well plate was filled with 50 µl of cell lysate and 50 µl of DNA lysis buffer per well and the standards in other wells. 100 µl of CyQuant GR dye, previously diluted 100 times with the DNA lysis buffer, was added to each well. After 20 minutes incubation, at room temperature in darkness, for color development, the fluorescence was measured with a Victor^3 1420 Multilabel Counter and excitation wavelength of 480 nm.

Luciferase assay: 100 µl of Luciferase assay reagent was added to 20 µl of cell lysate obtained from each transfected cells sample. Fluorescence was measured using the Victor3 1420 Multilabel Counter.

RT-qPCR Analysis: MG-63 cell lines were seeded at 5000 cells/cm^2. Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel) per manufacturer instructions. Quality of RNA was confirmed by loading 15 µl of diluted RNA solution with 15 µl of loading buffer on gel (1% agarose + 10 µl Ethidium Bromide). The cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) per manufacturer instructions and measured with MJMini. To test whether VEGF was expressed, RT-qPCR analysis was effectuated with a BIO RAD iCycler MyiQ machine measuring 55 cycles with temperature steps of 90°C, 60°C and 72°C.

Alamar blue and CDP-star assay: MG-63 cell lines were seeded at 4000 cells/cm^2. Cells were incubated in 1:10 Alamar Blue solution in darkness in basic medium for 4 hours after which fluorescence was measured with the Perkin Elmer Luninescence Spectrometer LS50G. Cells were then washed with PBS and a CDP-star assay (Roche) was performed per manufacturer instructions. Measurements were done with Victor3 1420 Multilabel Counter.

Statistical analysis; All experiments were performed in triplicate except otherwise stated. Each value represents the mean ± standard deviation (SD). In this investigation, the standard deviation (SD) was considered to be the standard uncertainty of the measurements. The significance (p<0.05) of differences among the groups was determined using ANOVA test.

Results

Effect of baicalein ($C_{15}H_{10}O_5$) *on angiogenic potential of MG-63 Cell Line;* to determine if baicalein ($C_{15}H_{10}O_5$) induces the expression of pro-angiogenic cytokines, HIF-1α activation and translocation, hypoxia responsive element (HRE) activation and VEGF expression were tested. To be a viable hypoxia mimic, baicalein ($C_{15}H_{10}O_5$) must stabilize HIF-1α and permit its translocation into the nucleus.

To visualize the HIF-1α, cells with different treatments (8hr and 24hr exposure to baicalein) were cultured along with a positive control (hypoxia). These cells were stained for HIF-1α, actin and the nucleus (Fig. 1). Cell exposed to longer period in baicalein developed similar morphologies to hypoxia-treated cells and the angular cell shape was similar to that found in some osteosarcomas cell lines. There was increased HIF 1α in the nucleus for longer exposure time as can be seen by comparing 8hr (Fig. 1A) with 24hr exposures (Fig. 1B) respectively. In both cases, HIF-1α staining was present in the nucleus just like in the positive hypoxia control (Fig. 1C). The images demonstrate that HIF-1α was stabilized and translocated into the nucleus under all experimental conditions, except for the negative control (data not shown).

To investigate if HIF-1α activates transcription of pro-angiogenic genes, a luciferase transfection assay was performed. A HRE-Luciferase vector was transfected into the cell and subjected to different conditions (4hr, 8hr and 24hr exposures in baicalein under normoxia) and (4hr, 8hr and 24hr exposures in hypoxia) with basic medium as the negative control and hypoxia as the positive control. Another triplicate wells were treated with baicalein and incubated in hypoxia for 24hr.

The resulting bioluminescence of each sample was corrected for the amount of cells by using CyQuant assay (Fig. 2). The transcription of the luciferase gene was significantly higher when baicalein was added, compared to basic and hypoxia conditions (p<0.05). It can also be seen that an increasing exposure time caused an increase of luminescence per DNA. Continuous exposure of baicalein in hypoxia generated the largest amount of luciferase. Similarly to hypoxia, baicalein increased the activation of the promoter of VEGF at a greater effect.

The localization of HIF-1α inside the nucleus and the increased luciferase expression due to exposure in baicalein prompted the question whether baicalein induced the expression of VEGF. To measure this, RT-qPCR was performed on duplicate samples of MG-63 cell line cultured under different baicalein exposure times (4hr, 8hr, 24hr) with basic medium as the negative control and hypoxia as the positive control. The

mean fold change of expression was calculated and is presented in Figure 2B. While the negative control showed no increased VEGF production, all other conditions resulted in significant increase in expression when compared to the negative control ($p<0.01$). Four (4) hr and 8hr baicalein exposure, and baicalein in hypoxia all had similar mean fold changes of VEGF.

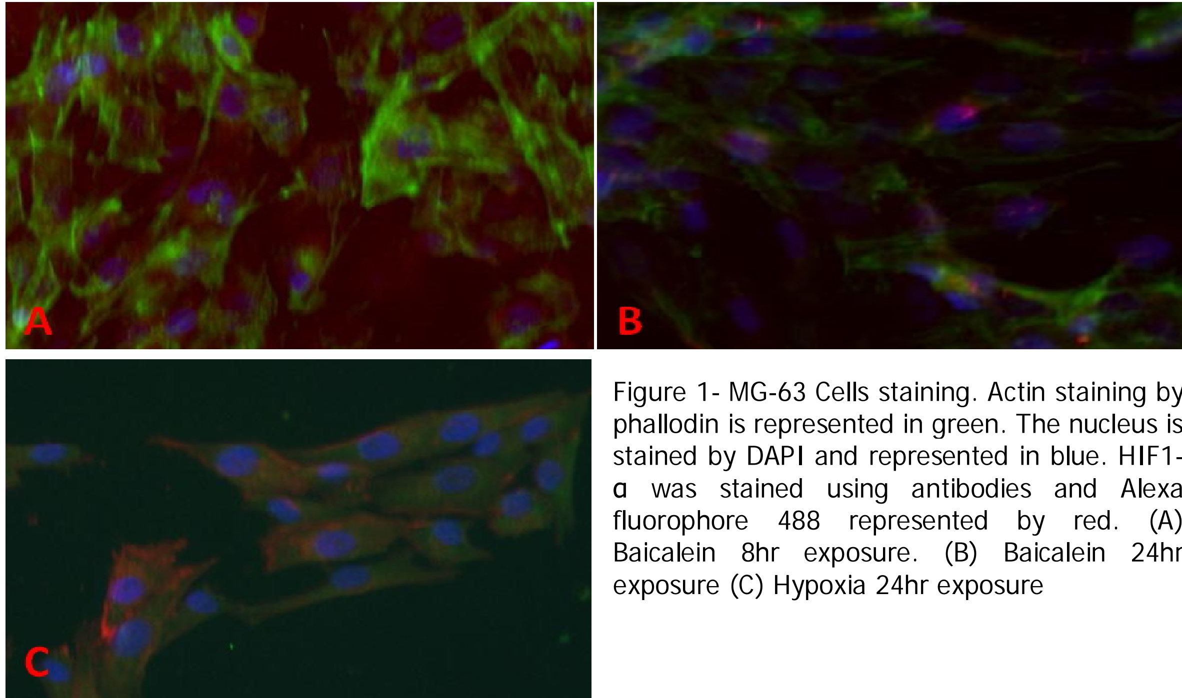

Figure 1- MG-63 Cells staining. Actin staining by phallodin is represented in green. The nucleus is stained by DAPI and represented in blue. HIF1-α was stained using antibodies and Alexa fluorophore 488 represented by red. (A) Baicalein 8hr exposure. (B) Baicalein 24hr exposure (C) Hypoxia 24hr exposure

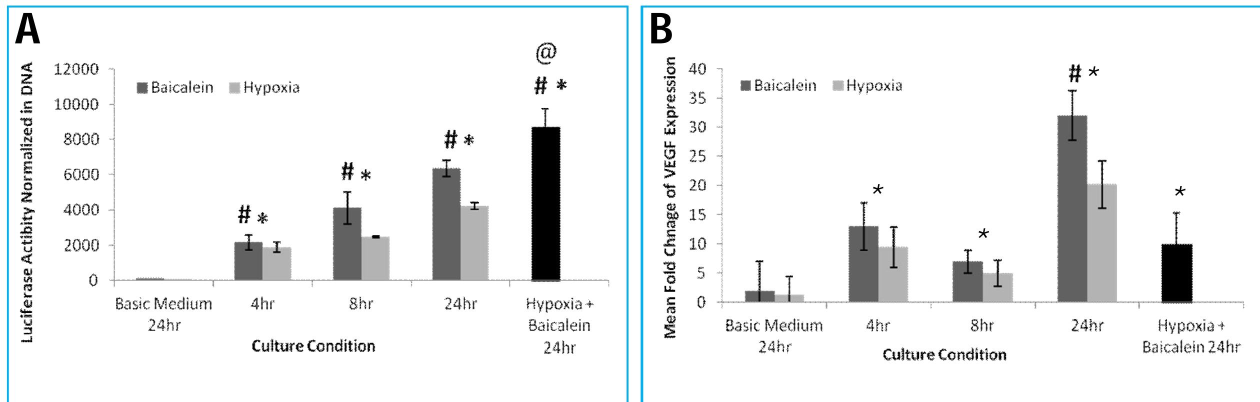

Figure 2- **(A)** Luciferase activity normalized for the DNA content **(B)** Mean fold change of VEGF compared to GAPDH expression by RT-qPCR all in 4hr, 8hr and 24hr following treatment in baicalein and hypoxia respectively. *, indicates significant difference from basic medium ($P<0.05$); #, indicates significant difference between conditions ($P<0.05$); @, indicates significant difference between baicalein+hypoxia and other conditions ($P<0.05$), n=5

Comparing all conditions, 24hrs exposure to baicalein showed a large increase in VEGF expression (3.5 fold increase, $p<0.01$). The overall tendency in the results suggests that baicalein has a similar or stronger effect on the expression of VEGF when compared to hypoxia. Baicalein showed an increased angiogenic capacity when compared to hypoxia. For baicalein to be a viable hypoxia mimicking agent it must also have a similar effect on osteogenic differentiation as hypoxia.

Effect of baicalein ($C_{15}H_{10}O_5$) *on osteogenic differentiation of MG-63 Cell Line;* To investigate the effect of baicalein on the osteogenic potential of MG-63 cell lines, ALP expression was measured using an Alamar blue/CDP-star assay to determine if baicalein can induce osteogenic differentiation. Using the CDP-star assay, the overall ALP level was measured and subsequently corrected with alamar blue for the amount of cells present (Fig. 3).

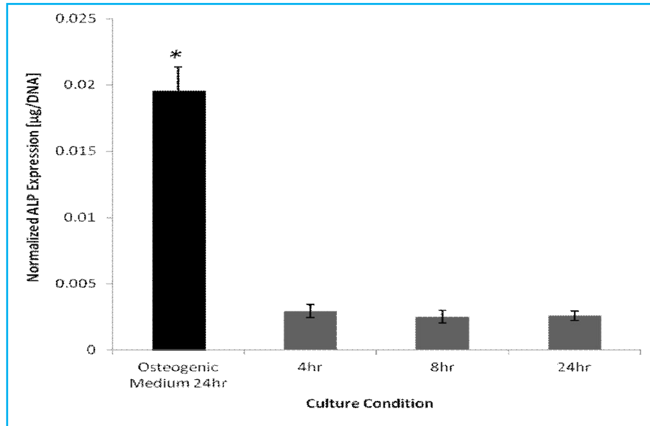

Figure 3- ALP expression normalized in DNA. MG-63 culture in osteogenic medium (without baicalein) under normoxic condition showed significant difference when compared to baicalein added osteogenic medium. n = 5

All treatments (4hr, 8hr, and 24hr in baicalein under) were cultured along with a positive control (osteogenic medium only). We found that baicalein had no significant osteogenic potential as it resulted in a far or lower ALP expression when compared to osteogenic medium. It is also important to note that in baicalein, ALP expression was independent of time of exposure. Since baicalein produced no osteogenic effect, there is a possibility that baicalein might inhibit osteogenesis of MG-63 cell lines.

Discussion

Hypoxia has been generally believed to be unfavorable on cells microenvironment that limits in vitro and in vivo cellular metabolism and growth (Baxi and Kaushal, 2008., 2008). Results show that exposure to baicalein has a similar and occasionally more enhanced effect than hypoxia treatment on production of angiogenic cytokines. The first experiment tested if baicalein had pro-angiogenic effects similar to hypoxia. One of the key steps in the production of many pro-angiogenic cytokines is the stabilization of HIF-1α. HIF-1α is remarkably unstable and is rapidly degraded by the proteasome pathway under normoxic condition. In contracts, hypoxia stables HIF-1α and activates transcription of target genes (Baxi and Kaushal, 2008). In this investigation, baicalein was found to stabilize HIF-1α and to enhance its translocation into cell nuclei independent of the exposure time. However, luciferase expression was found to be dependent on baicalein exposure time. The increases were significant compared to the luciferase expression induced by hypoxia.

Recent investigation revealed that HIF-1 binding sites are present in the VEGF gene promoter and that HIF-1 is required for hypoxia-induced VEGF gene transcription (Forsythe et al., 1996). In this investigation, we found that MG-63 cell line exposed in baicalein basic medium showed a clear increase in VEGF expression when compared to the negative control (basic medium without baicalein). The 24hr exposure to baicalein led to a significantly larger VEGF expression. The increased activation of hypoxia responsive elements found in previous results suggests that there should be an elevated response with longer exposure. But the present study, there was a decrease in expression when the exposure time was increased from 4hr and 8hr. Therefore the only conclusion obtainable from this last experiment is that baicalein has an effect on VEGF expression comparable to hypoxia.

Having demonstrated the viability of baicalein as a hypoxia mimicking agent in terms of angiogenic effects, the potential effect in tissue engineered bone grafts was evaluated by studying the effects of baicalein on osteogenic differentiation of MG-63 cell line. We found that baicalein had no significant osteogenic potential as it resulted in a lower ALP expression when compared to osteogenic medium without baicalein. However, it is important to note that the ALP expression is independent of time of exposure. Since baicalein produced no osteogenic effect, the possibility that baicalein might inhibit osteogenesis was also be tested. Baicalein, a major flavone of *Scutellariae baicalensis*, inhibits the 12-lipoxygenase (12-LOX) pathway of arachidonic acid metabolism, which inhibits cancer cell proliferation and induces apoptosis (Chen et al., 2013). Research shows that baicalein can selectively inhibit cancer cells while having significantly fewer inhibitory effects on normal ovarian cells (Chen et al., 2013). This may be a possible explanation of decreased expression of ALP in baicalein when compared to osteogenic medium.

Conclusion

In summary, this investigation revealed that baicalein could support increased HIF-1α-induced VEGF gene expression in osteosarcoma cell line (MG-63 cells), and thus is hypoxia mimicking agent. Further characterization of baicalein as hypoxia mimicking agent and its inhibitory effect on MG-63 cell line are needed, but current results appear promising.

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