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"MRGPRX2 - an important mast cell receptor to target itchy skin"

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1. Introduction

Sensitive skin is a prevalent concern worldwide, and its incidence continues to rise, with 73% of global consumers reporting increased skin sensitivity compared to previous years [1-2]. Sensitive skin encompasses a spectrum of conditions, including reactive skin, characterized by susceptibility to irritants and manifestations such as erythema, pruritus, burning, or stinging sensations. Atopic-prone skin exhibits an exaggerated immune response to stimuli, including allergens, environmental factors, and psychological stressors, culminating in dryness, pruritus, and inflammation [3]. Globally, 36% of consumers reporting sensitive skin identify pruritus as a primary symptom [1-2].

Mast cells, originating from hematopoietic stem cells in the bone marrow, are abundant immune cells in the skin and mucosa, functioning as a first line of defense against pathogens [3]. Within the skin, mast cells reside in close proximity to sensory nerve fibers, facilitating bidirectional communication. This interaction involves the release of mediators from mast cells, activating nerve fibers, and vice versa, contributing to pain and pruritus, among other sensations [4]. Mast cells express a diverse array of surface receptors, including Fc receptors, neuropeptide receptors such as the substance P receptor, complement receptors, and pathogen recognition receptors, all of which can trigger mast cell activation and degranulation. This study focuses on the Mas-related G protein-coupled receptor MRGPRX2, its inhibition, and its dual signaling pathways. MRGPRX2 is a G protein-coupled receptor (GPCR) associated with a heterotrimeric G protein (α, β, and γ subunits). Specifically, it couples to Gq/11, stimulating phospholipase C (PLC) activity. PLC hydrolyzes membrane phospholipids to generate inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), leading to an increase in intracellular calcium concentrations. Following G protein activation, G protein-coupled receptor kinases (GRKs) phosphorylate the receptor's cytoplasmic domain, promoting the recruitment of β-arrestin. This adapter molecule serves as a signaling scaffold for mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinases (ERKs), resulting in their phosphorylation and activation [4-6]. Mast cell degranulation results in the release of β-hexosaminidases, enzymes that catalyze the hydrolysis of hexosamine residues, such as glucosamine or galactosamine, from biomolecules, including oligosaccharides, glycoproteins, and glycolipids . These enzymes are typically localized within lysosomes. In mast cells, β-hexosaminidase is stored in granules and released extracellularly upon mast cell activation via exocytosis

(degranulation). Consequently, its release serves as a marker for mast cell activation, and degranulation. Fukuishi et al. [7] have demonstrated that β-hexosaminidase release is crucial for defense against bacterial invasion due to its ability to degrade peptidoglycans in bacterial cell walls.

Avena sativa (oat) is traditionally recognized for its phytomedicinal properties in soothing skin. This article describes a newly developed Avena sativa-derived active ingredient with enriched avenanthramide content, intended to alleviate pruritus. Avenanthramides comprise a group of approximately 40 to 50 structurally related amide esters consisting of various anthranilic and cinnamic acid partial structures [8]. Unique to oats, they provide phytoprotective benefits to the plant but are present in relatively low and variable amounts. The ingredient [INCI: Maltodextrin, Avena sativa (Oat) Kernel Extract] is an enriched oat extract standardized to 200-250 ppm of avenanthramides. It is manufactured using a specialized oat grain extraction process involving specific concentration and fractionation techniques employing adsorption-desorption principles.

Herein, we summarize our findings regarding the effects of this Avena sativa (oat) kernel extract on in vitro mast cell (MC) activation. The studies focus on the human mast cell line LAD2 and human skin mast cells, examining the impact of these compounds on degranulation, MRGPRX2 expression, downstream signaling, and calcium influx.

2. Materials and Methods

β-Hexosaminidase Release Assay for Mast Cell Degranulation

To determine MC degranulation, MCs were cultured for 24 h and pre-incubated with test substances for 2 h. After washing, cells were stimulated with compound 48/80 or substance P. A β-N-acetylglucosaminidase kit was used according to the manufacturer's instructions to evaluate the release rate of β-hexosaminidase from MCs (Merck, Germany). After harvesting the cells, the cell culture supernatant and MCs were collected for examination. MCs were sonicated and centrifuged to obtain a cell solution. A mixture containing 10 µL of sample (cell culture supernatant or cell solution) and 50 µL of substrate buffer was incubated at 37 °C for 15 minutes. The reaction was then stopped by adding alkaline solution, and the absorbance was measured at 400 nm. Each sample was incubated with the substrate at 37 °C for 1 min and hydrolyzed to produce p-nitrophenol. Hexosaminidase enzyme activity was calculated based on the premise that 1 µmol of p-nitrophenol corresponds to 1 unit of enzyme activity and was normalized to controls.

Analysis of ERK Phosphorylation in the Human MC Line LAD2 using Western Blot Analysis

MCs were cultured for 24 h and pre-incubated with test substances for 2 h and then stimulated with compound 48/80 for 10 min. Cells were harvested and lysed. For normalization, protein content in cell lysates was determined using a bicinchoninic acid (BCA) protein assay kit. Laemmli buffer was added to lysates, and these samples were boiled at 95 °C for 5 min before separation on 10-12.5% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes (neoLab Migge Laborbedarf-Vertriebs GmbH, Germany). Membranes were blocked in 5% w/v BSA (Sigma-Aldrich) in TBST and then incubated with primary antibodies diluted 1:1000 overnight at 4 °C. The following day, membranes were incubated with goat-anti-rabbit-HRP or goat-anti-mouse-HRP secondary antibodies (1:10,000, Dianova, Germany) before

detection with WesternBright Quantum ECL HRP substrate (advansta, Menlo Park, USA) using a Fusion-FX7 (Vilber Lourmat, Germany). Primary antibodies used were anti-GAPDH rabbit mAb (#2118, Cell Signaling Technology), anti-phospho-ERK (Ser463/465) rabbit mAb (#9516, Cell Signaling Technology), and anti-ERK rabbit mAb (#6944, Cell Signaling Technology)

Measurement of Ca^{2+} Influx in MCs using Flow Cytometry

LAD2 cells or skin MCs were pre-exposed to Avena sativa (oat) kernel extract (100 ppm) for 2 h. Cells were then loaded with the Ca^{2+}-sensitive dye Indo 1 AM. One volume of aqueous Indo-1 AM ester dispersion was added to one volume of cell suspension. Cells were incubated for 30 minutes at 37 °C, washed, and incubated for a further 30 minutes to allow for complete de-esterification of intracellular AM esters. Cells were then mounted and measured for 30 seconds in a flow cytometer to determine baseline fluorescence. Subsequently, c48/80 was added, and cells were measured for 5 min. Finally, ionomycin was added, and measurements were taken for 2 min to determine the maximal Ca^{2+} fluorescence.

Detection of Surface LAMP-1 and CD63

Mast cells were treated with Avena sativa (oat) kernel extract (100 ppm) or buffer control for 2 h, followed by stimulation with substance P or compound 48/80. Primary antibody was added for 1 h, followed by fixation in an equal volume of PBS containing 2.2% formaldehyde for 30 min. Cells were washed three times with PBS/bovine serum albumin and incubated with FITC-conjugated secondary antibody for 30 min. After washing and resuspending, 10,000 cells were analyzed for each study using an Epics Flow Cytometer (Coulter, Hialeah, FL), gated to exclude debris.

MRGPRX2 Activation Assay

The PathHunter® β-Arrestin assay monitors the activation of the GPCR MRGPRX2 receptor using a homogeneous, non-imaging assay format based on Enzyme Fragment Complementation (EFC) with β-galactosidase (β-Gal) as the functional reporter. The enzyme is split into two inactive complementary portions: a small peptide (ProLink™) and a larger protein (Enzyme Acceptor; EA). PK and EA are expressed as fusion proteins in the cell, with PK fused to the GPCR of interest and EA fused to β-Arrestin. Activation of the target GPCR and recruitment of β-Arrestin leads to PK and EA complementation, restoring β-Gal activity, which is measured using chemiluminescent PathHunter® Detection Reagents.
PathHunter cell lines were expanded from freezer stocks according to standard procedures. Cells were seeded into white-walled, 384-well microplates and incubated at 37°C for an appropriate period before testing. For agonist determination with substance P, cells were incubated with sample to induce response. The sample was added to cells and incubated at 37°C for at least 120 minutes. The final assay vehicle concentration was 1%. For antagonist determination with the ingredient, cells were pre-incubated with the ingredient in a dose-dependent manner for 30 min, followed by substance P challenge at the EC80 concentration for at least 120 minutes. Assay signal was generated via addition of PathHunter® Detection reagent cocktail, followed by a one-hour incubation at room temperature. Microplates were read following signal generation with a PerkinElmer Envision™ instrument for chemiluminescent signal detection. Ingredient activity was analyzed using CBIS data analysis suite (ChemInnovation, CA).

3. Results

Avena sativa (oat) kernel extract, later referred as the ingredient (or extract), reduces β-hexosaminidase release induced by several known MRGPRX2 activators including compound 48/80, substance P and cortistatin 14 in LAD2 and primary skin mast cells (Figure 1). Lysosomal-associated membrane protein 1 (LAMP-1) and CD63 are membrane proteins localized in cellular lysosomes and mast cell granules. LAMP-1 is involved in maintaining lysosomal integrity, pH and catabolism. CD63 belongs to the tetraspannin protein family and occurs in intracellular membranes including lysosomes. It is involved in several signaling processes eg. Apoptosis, adhesion and cytoskeleton organisation. After mast cell granule exocytoses and thereby fusion of lysosomal membrane with the plasma membrane, LAMP1 and CD63 are localized at the cell surface of degranulated mast cells and are therefore used as degranulation markers. Here, LAMP-1 and CD63 staining substantiates the inhibitory effect of the ingredient on substance P and cortistatin-14 induced mast cell degranulation (Figure 2).

Here, we clarify the molecular mechanism of the effects of the ingredient on mast cell signaling to elaborate how it reduces mast cell degranulation and therewith inhibits itching sensation. Thereby, we start at receptor level with MRGPRX2 activation studies (Figure 3) and then we demonstrate its effect on down stream signaling such as Calcium influx (Figure 4) and ERK phosphorylation (Figure 5).

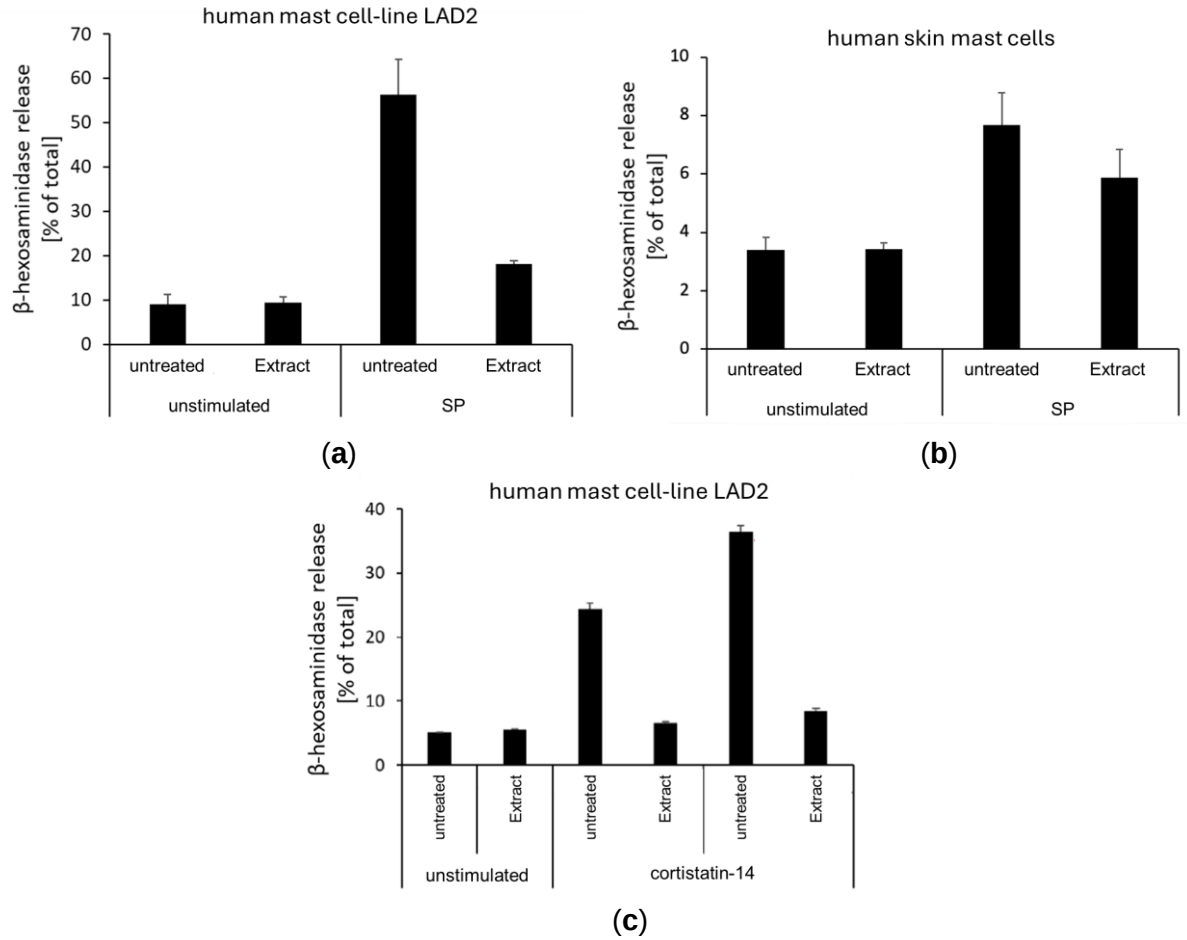
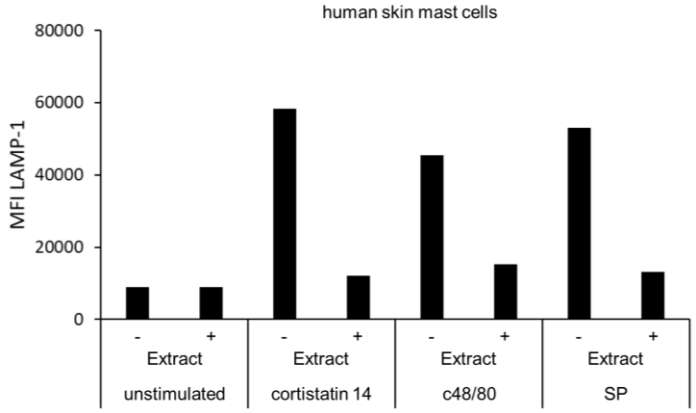
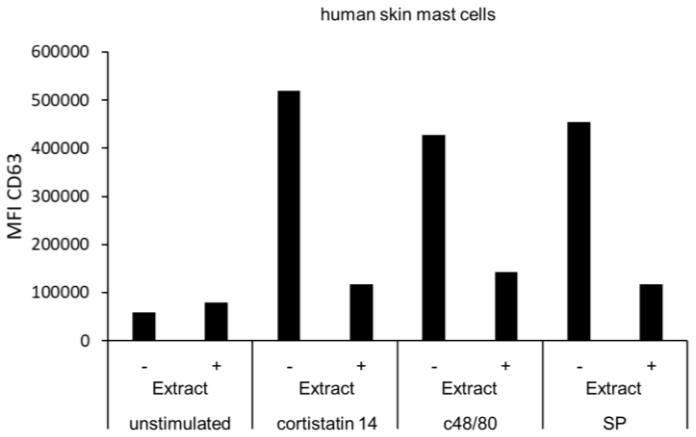


Figure 1.: Graphical Illustration of mast cell degranulation determined by hexosaminidase activity that is released from granules upon stimulation with SP and Cortistatin.14. The

ingredient reduces both Cortistatin-14 and Substance P induced upregulation of hexosaminidase release in LAD2 and primary skin MCs.

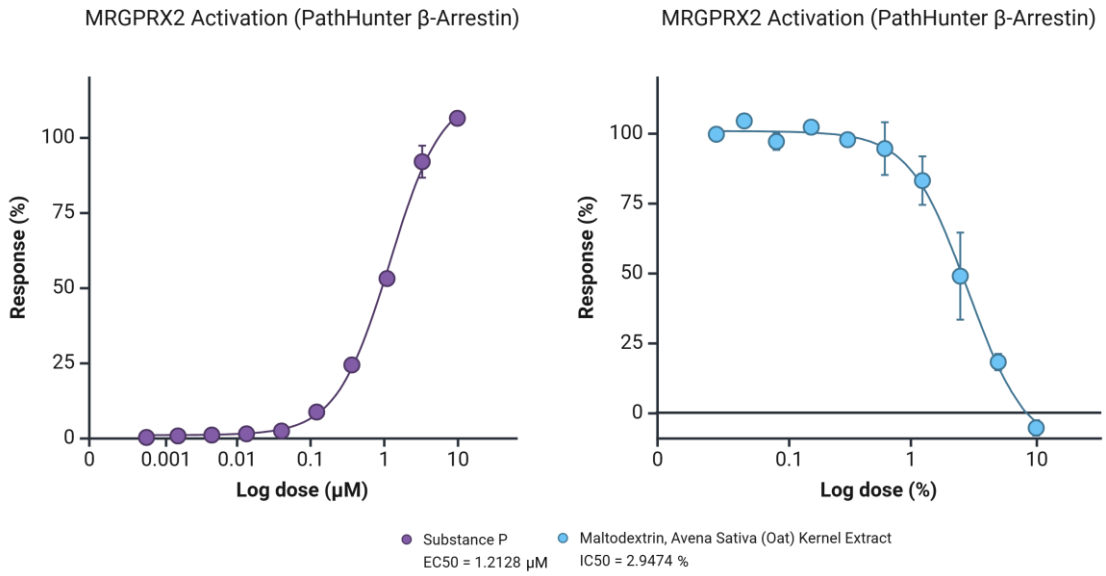


(**a**)



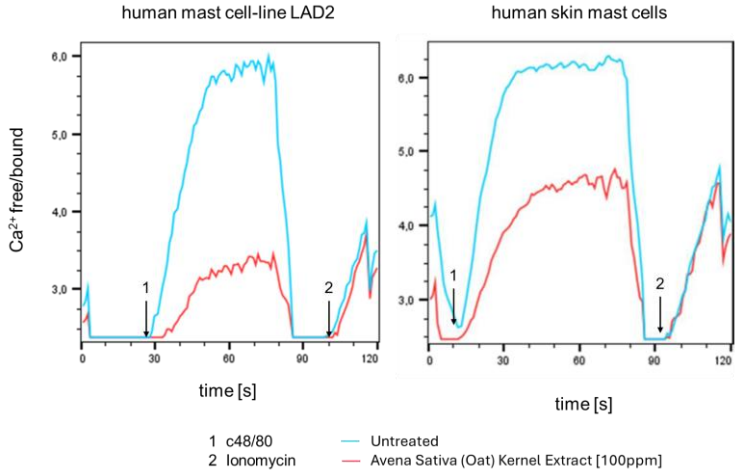
(**b**)

Figure 2.: Bar graphs represent the LAMP-1 and CD63 staining in response to cortistatin-14, c48/80 and substance P as MC degranulation markers. Human primary skin mast cell stimulation substantially increases LAMP-1 and CD63 surface expression which is in turn inhibited by the extract.



(**a**) (**b**)

Figure 3.: Control dose response curve for the selected GPCR Biosensor Assay Control dose curve was performed for the requested GPCR Biosensor Assay. Data shown was normalized to the maximal and minimal response observed in the presence of control compound and vehicle respectively. For antagonist assay, data was normalized to the maximal and minimal response observed in the presence of EC80 ligand and vehicle.



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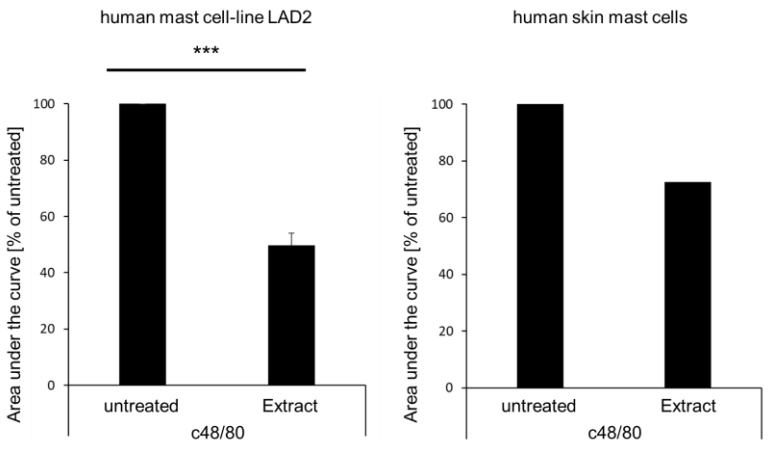


Figure 4.: Detection of Calcium influx: Pre-exposure to the extract 100 ppm for 2h and stimulation with c48/80 followed by Ca2+ measurement using flow cytometry.

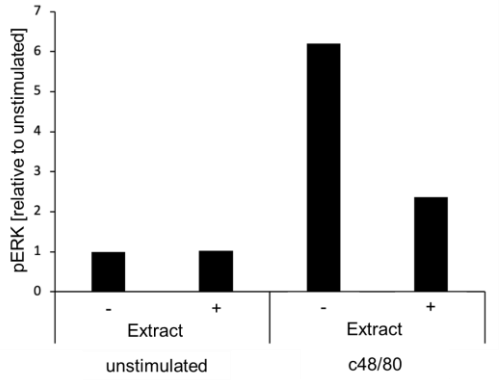
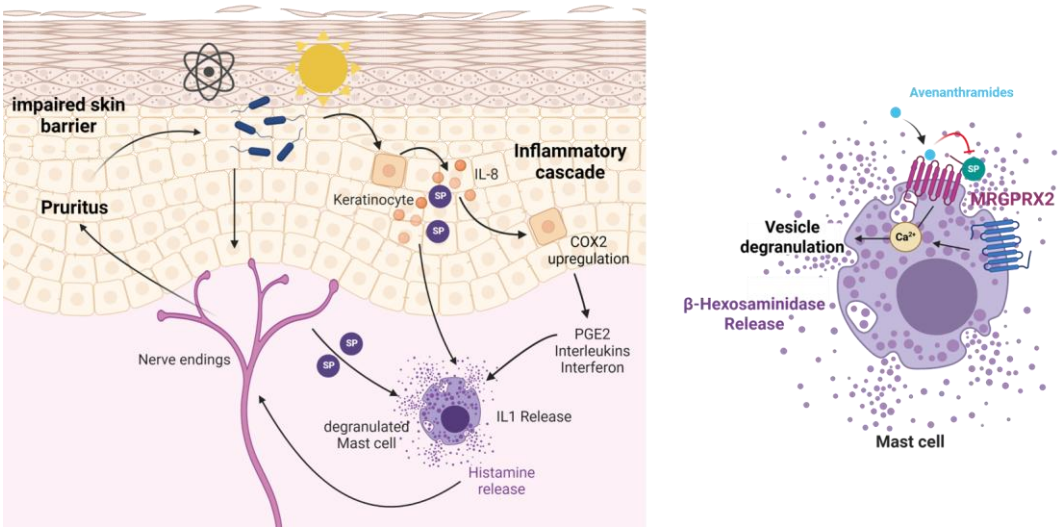


Figure 5.: Erk phosphorylation detected by Western Blot analysis in LAD2 MCs.

4. Discussion and Conclusion

The study suggests that the ingredient has significant potential as an inhibitor of mast cell activation. It consistently reduces degranulation, modulates MRGPRX2 induced signaling, and diminishes calcium influx and ERK phosphorylation. Thereby receptor studies have shown that the ingredient already inhibits these effects at the level of the MRGPRX2 receptor. Here, we conclude that the ingredients lead compounds – avenanthramides – bind to the receptor thereby deminishing agonists to bind and activate the receptor (Figure 6b). These findings support further investigation into the use of the ingredient as a therapeutic agent for mast cell-related disorders including pruritus (Figure 6a).



(**a**) (**b**)

Figure 6.: (a) Role of Mast Cells in skin sensitivity and pruritus perception. (b) Mechanism of action of avenanthramides standardized in Avena sativa (oat) kernel extract inhibiting MRGPRX2 receptor and subsequent signaling and pruritus generation upon stress.

5. References

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