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## Antimicrobial activity of apitoxin, melittin and phospholipase A<sub>2</sub> of honey bee (*Apis mellifera*) venom against oral pathogens

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### ABSTRACT

In this work, we used the Minimum Inhibitory Concentration (MIC) technique to evaluate the antibacterial potential of the apitoxin produced by *Apis mellifera* bees against the causative agents of tooth decay. Apitoxin was assayed *in natura* and in the commercially available form. The antibacterial actions of the main components of this apitoxin, phospholipase A<sub>2</sub>, and melittin were also assessed, alone and in combination. The following bacteria were tested: *Streptococcus salivarius*, *S. sobrinus*, *S. mutans*, *S. mitis*, *S. sanguinis*, *Lactobacillus casei*, and *Enterococcus faecalis*. The MIC results obtained for the commercially available apitoxin and for the apitoxin *in natura* were close and lay between 20 and 40 µg / mL, which indicated good antibacterial activity. Melittin was the most active component in apitoxin; it displayed very promising MIC values, from 4 to 40 µg / mL. Phospholipase A<sub>2</sub> presented MIC values higher than 400 µg / mL. Association of melittin with phospholipase A<sub>2</sub> yielded MIC values ranging between 6 and 80 µg / mL. Considering that tooth decay affects people's health, apitoxin and its component melittin have potential application against oral pathogens.

**Key words:** antibacterial activity, apitoxin, Melittin, Phospholipase A<sub>2</sub>, tooth decay, oral pathogens.

### INTRODUCTION

More than 700 bacterial species have been detected in the oral cavity. Some of these bacteria have been implicated in oral diseases such as caries and periodontitis, which are among the most common bacterial infections in humans (Aas et al. 2005).

Tooth decay is normally associated with *Streptococcus* spp., especially *S. mutans* and *S. sobrinus*, and *Lactobacillus* spp. (Hirasawa and Takada 2002, Chung et al. 2006). This

disease has a negative impact on people's health, because it diminishes the mastication function, alters the psychosocial development and facial aesthetics, causes phonetic disturbances and pain, and generates local and systemic infectious complications (Melo et al. 2008).

On the basis of the effects that tooth decay exerts on humans, it is important to find other sources of promising compounds that can act against the causative agents of this disease. Most of the commercially available pharmaceuticals consist of direct or indirect derivatives of plants,

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microorganisms, marine organisms, and vertebrate and invertebrate terrestrial animals (Chin et al. 2006). Historically, the use of plants as medicines has been extensively acknowledged, studied, and reviewed. However, animals have not received the same attention as potential source of medicinally relevant substances, even though insects constitute an excellent example of this potential. When the amount of research per species is compared, chemicals present in plants have been 7,000 times more frequently investigated than those existing in insects. In recent years, insects have increasingly attracted researchers' interest, contributing to new discoveries (Trowell 2003, Alves and Alves 2011). In this sense, it is relevant to investigate insects as a potential source of new substances that can help to combat tooth decay.

Bees belonging to the species *Apis mellifera* participate in a number of activities that are closely related to human beings. Traditional examples are pollination and the production of honey, resins, wax, propolis, royal jelly, pollen, and apitoxin (bee venom). Compounds synthesized by bees have been extensively studied due to their various therapeutic applications (Varanda and Tavares 1998, Perumal Samy et al. 2007, Boutrin et al. 2008, Alia et al. 2013).

Among the substances produced by bees, apitoxin is one of the most important. Glands located in the abdomen of these insects synthesize this complex chemical. Apitoxin consists of 88% water; the remaining 12% contains components such as hyaluronidase, phospholipase A<sub>2</sub>, histamine, melittin, and some other peptides like apamin, secapin, among others (Lima and Brochetto-Braga 2003, Alia et al. 2013).

Concerning the apitoxin components, phospholipase A<sub>2</sub> has been one of the most extensively investigated compounds. According to Samel et al. (2013), phospholipases A<sub>2</sub> are enzymes that catalyze the hydrolysis of the *sn*-2 fatty acyl ester bond of *sn*-3 phosphoglycerides, liberating free fatty acids, and lysophospholipids.

Phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) are a large family of proteins found in various mammalian tissues, arthropods, and in the venoms of snakes, scorpions, and bees. On the basis of their source, catalytic activity, amino acid sequence, chain length, and disulfide bond patterns, PLA<sub>2</sub>s are divided into 16 groups (Murakami et al. 2011) including 10 groups of secretory PLA<sub>2</sub>s (sPLA<sub>2</sub>s) (Schaloske and Dennis 2006, Burke and Dennis 2009).

Phospholipase A<sub>2</sub> has low molecular weight, large immunogenic potential, and high catalytic activity. This enzyme displays antibacterial and anticoagulant actions and plays an active role in the generation of chemical mediators, cell proliferation, muscle contraction, and anti-inflammatory processes (Van Deenen and De Haas 1963, Nevalainen et al. 2008).

Melittin is another fundamental component of apitoxin. It comprises 26 amino acid residues with amphipathic characteristics (polar and non-polar ends). These residues allow melittin to interact with lipid membranes, and to increase the permeability of the erythrocytes and other cell membranes. These amino acids constitute around 50% of the apitoxin of bees belonging to the species *Apis mellifera* (Lima and Brochetto-Braga 2003). Melittin is cytotoxic and has potential action in cell lysis, as evidenced for human erythrocyte lysis (Pandey et al. 2010) as well as other peptides. Furthermore, it acts directly on the cell membrane (Zhu et al. 2007, Carvalho and Machini 2013). Several biological activities have been attributed to melittin, including antibacterial, antiviral, and anti-inflammatory actions, cell growth inhibition, and apoptosis of different cancer cell lines (Raghuraman and Chattopadhyay 2007, Wang et al. 2009, Alia et al. 2013).

Bearing in mind the numerous biological properties reported for apitoxin and its derivatives, we aimed to evaluate the antibacterial potential of the apitoxin of *Apis mellifera* bees, both *in natura* and in its commercially available form, against pathogens that cause tooth decay. We also assessed

the antibacterial action of the major components of this apitoxin, phospholipase A<sub>2</sub> and melittin, alone and in combination.

## MATERIALS AND METHODS

### COMMERCIAL APITOXIN

Commercially available apitoxin was supplied by Cooperativa Nacional de Apicultores (National Cooperative of Beekeepers, Conap), situated in the city of Belo Horizonte, state of Minas Gerais, Brazil, manufacturing batch AP/009. This apitoxin was collected by electric impulse. An electric shock stimulated the bees to sting a film covering a glass plate, where the venom was collected.

### COLLECTION OF APITOXIN IN NATURA BY MANUAL EXTRACTION

Apitoxin was collected in the research laboratory of the Bees Sector of the Department of Genetics, Ribeirão Preto Medical School, University of São Paulo (FMRP-USP), Brazil. *Apis mellifera* bees were collected from the hive, placed in a small box, and stored at -18°C in a freezer, until the insects became motionless. With the aid of a stereomicroscope, the sting apparatus was totally removed from the bees by using a pair of tweezers with fine tips. Then, the venom reservoir was pressed, and the venom was collected inside a 20-μL microcapillary (Blaubrand®). The ends of the microcapillary were sealed with bee wax, to avoid evaporation, and the samples were immediately sent to the Laboratory of Research in Applied Microbiology (LaPeMA) of the University of Franca, Franca, state of São Paulo, Brazil, for accomplishment of the antibacterial assays.

### APITOXIN COMPONENTS

Melittin (code M2272-5MG) and phospholipase A<sub>2</sub> (code P9279-5MG) were purchased from Sigma-Aldrich®, manufacturing batches 038K4061 and 078K4102, respectively.

### PREPARATION OF APITOXIN, PHOSPHOLIPASE A<sub>2</sub>, MELITTIN, AND ASSOCIATION OF THE MAJOR COMPONENTS

One milligram of apitoxin *in natura*, commercial apitoxin, melittin, and phospholipase A<sub>2</sub> were used to determine the minimum inhibitory concentration (MIC). To test the association of phospholipase A<sub>2</sub> with melittin, 0.5 mg of each compound was employed.

### TESTED MICROORGANISMS

The bacteria were acquired from the American Type Culture Collection (ATCC) maintained in the culture collection of the Laboratory of Research in Applied Microbiology (LaPeMA) of the University of Franca, state of São Paulo, Brazil, at -80°C. The following microorganisms were used: *Streptococcus salivarius* (ATCC 25975), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49452), *Streptococcus sanguinis* (ATCC 10556), *Lactobacillus casei* (ATCC 11578), and *Enterococcus faecalis* (ATCC 4082).

### ANTIMICROBIAL ASSAYS

The MIC values for commercial apitoxin, apitoxin *in natura*, melittin, phospholipase A<sub>2</sub>, and the association of melittin with phospholipase A<sub>2</sub> were determined by the broth microdilution method in 96-well microplates, according to the methodology described by CLSI (2006). The samples were dissolved in 125μL of tryptic soy broth (TSB), to yield concentrations of the tested compounds between 4 and 400μg/mL. The inoculum was adjusted at 625nm for each microorganism in a spectrophotometer, to obtain a cell concentration of 5 x 10<sup>5</sup> colony-forming units (CFU/mL) (CLSI 2006). Chlorhexidine digluconate (Sigma-Aldrich) at concentrations varying from 0.01 to 5.9μg/mL was employed as the positive control. The microplates were incubated at 37°C for 24h, and then 30μL of 0.02% resazurin (Sigma-Aldrich) aqueous solution was added to each well (Sarker et al. 2007). Resazurin is an oxireduction probe that allows for

immediate observation of microbial growth. The blue and red colors represent the absence and the presence of microbial growth, respectively.

## RESULTS AND DISCUSSION

Oral diseases affect 3.9 billion people worldwide (Marcenes et al. 2013). Dental treatment is often very expensive and not readily accessible, especially in developing countries (More et al. 2008). In this sense, extensive efforts have been made toward the search for anticariogenic compounds that can be incorporated into dental products (Cai and Wu 1996, Ambrosio et al. 2008).

Several antimicrobials, such as ampicillin, chlorhexidine, sanguinarine, metronidazole, phenolic antiseptics, and quaternary ammonium antiseptics, among others, effectively prevent dental caries (Chung et al. 2006, Tsui et al. 2008). However, various adverse effects such as tooth and restoration staining, increased calculus formation, diarrhea, and disarrangements of the oral and intestinal flora have been associated with the use of these chemicals (Chung et al. 2006, More et al. 2008). These drawbacks justify the search for new effective anticariogenic compounds that could aid caries prevention (Porto et al. 2009).

Here, we evaluated the action of apitoxin in natura, commercial apitoxin, and the major components of apitoxin, melittin and phospholipase A<sub>2</sub>, alone or in combination, against the bacteria that cause tooth decay. We obtained MIC data for all the assayed microorganisms. During the experiments, we did not detect any contamination by fungi or other bacteria. We considered that MIC values higher than 400 µg / mL corresponded to lack of antibacterial action, because this was the highest concentration tested in this study. Concerning the antimicrobial assays of compounds isolated from natural sources, some authors (Rios and Recio 2005, Gibbons 2008) have established MIC value criteria for determination of their antimicrobial potential. These authors suggested that MIC values lower than 100.0 µg/mL are considered very promising in the search for new anti-infection agents.

Table I lists the MIC data for apitoxin in natura, commercial apitoxin, melittin, phospholipase A<sub>2</sub>, and the association of melittin with phospholipase A<sub>2</sub> obtained after accomplishment of the microdilution broth technique. The MIC values varied from 4 to more than 400 µg/mL in the presence of the tested microorganisms (Table I).

Apitoxin in natura afforded MIC values for all the assayed bacteria: 20 µg/mL for *S. salivarius*, *E. faecalis*, *L. casei*, and *S. mutans*; 30 µg/mL for *S. sanguinis*; and 40 µg/mL for *S. sobrinus* and *S. mitis*. Commercial apitoxin presented MIC values of 20 µg/mL for all the investigated bacteria. Compared with apitoxin in natura, commercial apitoxin was more efficient against *S. sanguinis*, *S. sobrinus*, and *S. mitis*, culminating in lower MIC values. The results achieved for the two apitoxins were close, so both of them exhibited a good antibacterial activity.

Melittin alone was the most active component. It displayed promising MIC values: 4 µg/mL for *L. casei*; 10 µg/mL for *S. salivarius*, *S. sanguinis*, *S. sobrinus*, and *S. mitis*; 6 µg/mL for *E. faecalis*, and 40 µg/mL for *S. mutans*. As for phospholipase A<sub>2</sub> alone, it did not yield any satisfactory results; the MIC value was 400 µg/mL for *L. casei*, and it did not display antibacterial activity against any of the other tested microorganisms. The association of melittin and phospholipase A<sub>2</sub> resulted in MIC values of 6 µg/mL for *L. casei*; 8 µg/mL for *E. faecalis*; 10 µg/mL for *S. salivarius*, *S. sanguinis*, *S. sobrinus*, and *S. mitis*; and 80 µg/mL for *S. mutans*.

Perumal Samy et al. (2007) investigated the antibacterial activity of a series of animal venoms, including the apitoxin of *Apis mellifera*, against the bacteria *Escherichia coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, *P. mirabilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* by the diffusion agar method. Apitoxin was active; 23.2 mm inhibition halos originated in the case of the Gram-positive bacterium *S. aureus*, but this toxin had no effect on Gram-negative bacteria.

TABLE I  
*In vitro* antibacterial activity (MIC) from apitoxin *in natura*, comercial apitoxin, melittin, phospholipase A<sub>2</sub>, and association of melittin with phospholipase A<sub>2</sub> against oral pathogens.

Microorganism	Minimum Inhibitory Concentration (µg / mL)					Control Chlorhexidine
	Apitoxin in natura	Commercial apitoxin	Melittin	Phospholipase A <sub>2</sub>	Association Melittin/ Phospholipase A <sub>2</sub>	
<i>S. salivarius</i> (ATCC 25975)	20.0	20.0	10.0	-*	10.0	0.9
<i>E. faecalis</i> (ATCC 4082)	20.0	20.0	6.0	-	8.0	3.7
<i>L. casei</i> (ATCC 11578)	20.0	20.0	4.0	400.0	6.0	0.9
<i>S. sanguinis</i> (ATCC 10556)	30.0	20.0	10.0	-	10.0	3.7
<i>S. sobrinus</i> (ATCC 33478)	40.0	20.0	10.0	-	10.0	0.9
<i>S. mitis</i> (ATCC 49452)	40.0	20.0	10.0	-	10.0	3.7
<i>S. mutans</i> (ATCC 25175)	20.0	20.0	40.0	-	80.0	0.9

\* > 400µg / mL corresponds to lack of antibacterial activity.

Our study confirmed this activity against Gram-positive bacteria: both commercial apitoxin and apitoxin *in natura* presented excellent antibacterial activity against the Gram-positive bacteria that cause tooth decay.

Kim et al. (2006) employed MIC and Minimum Bactericidal Concentration (MBC) to assess the activity of apitoxin against *S. mutans* (ATCC 25175); they obtained a value of 64µg/mL in both cases. Here, we also used *S. mutans*, one of the main cariogenic bacteria, that participates in the onset of the tooth decay process (Chung et al. 2006). Both commercial apitoxin and apitoxin *in natura* yielded a MIC value of 20µg/mL. This value was lower than that found by Kim et al. (2006), who revealed the antibacterial potential of apitoxin.

Some studies have reported that the association of melittin with phospholipase A<sub>2</sub> enhances the activity of the former compound. This is because this association generates a complex that acts mainly on the plasma membrane, to culminate in cell lysis. In contrast, the results from our experiments did not suggest that melittin and phospholipase

A<sub>2</sub> interacted effectively (Mollay and Kreil 1974, Mollay et al. 1976). Although the association of these compounds presented excellent antibacterial activity, with MIC values lying between 6 and 80µg/mL, phospholipase A<sub>2</sub> was not able to significantly enhance the antibacterial action of melittin alone (which ranged between 4 and 40µg/mL). Therefore, these data led us to infer that it was the action of melittin that inhibited bacterial growth.

Perumal Samy et al. (2007) assessed the antibacterial activity of phospholipase A<sub>2</sub> purified from the venom of *Apis mellifera* by the agar diffusion method. Like apitoxin, phospholipase A<sub>2</sub> generated 13.3 mm inhibition halos only against the Gram-positive bacterium *S. aureus*. In the present work, phospholipase A<sub>2</sub> provided MIC values of 400µg/mL against *L. casei*, but it was not active against any of the other assayed bacteria. Therefore, this compound did not constitute a promising antibacterial agent against the microorganisms that cause tooth decay.

Boutrín et al. (2008) incubated bacterial cultures of *Enterobacter cloacae*, *Citrobacter*



*freundii*, and *E. coli* with various concentrations of the enzyme phospholipase A<sub>2</sub>. The effects of this enzyme on the bacteria depended on the enzyme concentration, bacterial exposure, and bacterial growth phase. These authors showed that these bacteria were equally sensitive to phospholipase A<sub>2</sub> at 2h of culture. However, after 12h, the microorganisms had different susceptibilities to this enzyme, namely *E. cloacae* < *C. freundii* < *E. coli*. In our study, the bacterial inoculum was prepared with microorganisms that had been cultured for 24h, so it was not possible to establish whether the microorganisms were in the early or late exponential growth phase. For this reason, it was difficult to explain the lack of antibacterial effect for phospholipase A<sub>2</sub> at the tested concentrations (4 to 400µg/mL).

The bacterial envelope sites engaged in cell growth may represent preferential sites for phospholipase A<sub>2</sub> to act against Gram-positive bacteria (Foreman-Wykert et al. 1999). To exert its antibacterial activity, phospholipase A<sub>2</sub> must first bind and traverse the bacterial cell wall, to produce the extensive phospholipid membrane degradation required for bacterial killing. Phospholipid hydrolysis and bacterial killing become more effective upon addition of sublethal doses of β-lactam antibiotics to the bacterial cultures (Foreman-Wykert et al. 1999, Koduri et al. 2002). Moreover, bacteria are more resistant against the bactericidal action of phospholipase A<sub>2</sub> when they are in the stationary phase rather than in the logarithmic (growth) phase, suggesting that these microorganisms are more susceptible to phospholipase A<sub>2</sub> when they are dividing (Foreman-Wykert et al. 1999, Koduri et al. 2002). Overall, bacterial cell wall components outside the phospholipid membrane seem to bar the access of phospholipase A<sub>2</sub> to the phospholipid membrane surface (Koduri et al. 2002). Phospholipase A<sub>2</sub> can act on Gram-positive bacteria through several other possible sites (Foreman-Wykert et al. 2000, Beers

et al. 2002, Ghomashchi et al. 1998, Koduri et al. 2002). However, according to Boutrin et al. (2008), this enzyme cannot be directly applied to Gram-negative bacteria because their cell wall structures differ from those of Gram-positive microorganisms, in particular with respect to the presence of the outer membrane, which protects the cytoplasmic membrane. The interaction of phospholipase A<sub>2</sub> with bacterial lipopolysaccharide (LPS) might expose the inner leaf of the outer membrane to the action of the enzyme, facilitating its penetration into the cytoplasmic membrane.

Using MIC values, Alia et al. (2013) examined the antimicrobial activity of melittin isolated from the venom of *Apis mellifera* against the Gram-positive *S. aureus* (ATCC 11632) and *Listeria monocytogenes* (ATCC 19111), and the Gram-negative *Salmonella enterica* (ATCC 7001) and *Yersinia kristensenii* (ATCC 33639). MIC was 12.5µg/mL for *L. monocytogenes*, 25µg/mL for *S. aureus*, 100µg/mL for *S. enterica*, and 200µg/mL for *Y. kristensenii*. Melittin afforded the best results against the Gram-positive bacteria, as we also verified in our study. Here, melittin alone gave the most promising results: MIC values varying between 4 and 40µg/mL against the causative agents of tooth decay.

Melittin is an antimicrobial peptide (AMP). AMPs are components of innate immunity and occur in virtually every kingdom and phylum, which attests to their role in primitive immune response (Andreu and Rivas, 1998). They are defined as peptides of 12–50 amino acids in length, with a molecular mass of less than 10kDa. They bear a net positive charge ranging from +2 to +7, because the number of basic amino acids (arginine, lysine, and histidine) exceeds the number of acidic amino acids (aspartate and glutamate). Generally, 50% or more of the AMP amino acids are hydrophobic. Indeed, such peptides interact with bacterial membranes as part of their mechanism of action (Hancock and Diamond 2000, Teixeira et al. 2012, Adade et al. 2013).

AMPs can potentially kill a broad range of microorganisms, including Gram-negative and Gram-positive bacteria, fungi, protozoa, and viruses. Their ability to kill microbes, stems from their cationic charge and their peptide structure. Due to their positive charge, AMPs are attracted to anionic components present on the surface of the lipid membranes of these microorganisms (Kagan et al. 1994, Izadpanah and Gallo 2005). This initial electrostatic attraction leads to nonspecific association of the AMP with the target membrane. The structure of both LL-37 and  $\beta$ -defensins is organized in such a way that they have a hydrophobic side opposed to their cationic side. This amphipathic structure drives AMP penetration through the membrane. Hence, the specific microbicidal properties of AMPs are related to the ability of each of these structures to bind and assemble in the target membrane. Many mechanisms can disrupt the membrane, including pore formation or a detergent-like solubilization. This change in membrane permeability dissipates ion gradients and energy, which culminates in cell lysis within minutes. Host cells are resistant to lysis because their lipid composition differs from those of the target cells for which AMPs have evolved to kill (Izadpanah and Gallo 2005).

The discovery of the role played by plant- and animal-derived AMPs in the fight against microbial infections has led researchers to propose that they might be the basis for a new class of clinical antimicrobials (Wiesner and Vilcinskas 2010). According to Carvalho and Machini (2013), AMPs have some advantageous properties: easy metabolism, low microbial resistance, microbicidal action, synergistic effects with other antibiotic drugs, and broad action spectrum. Hence, investigations into AMPs shall increase the current knowledge about drugs and aid in design of novel pharmaceuticals. Unfortunately, AMPs also present drawbacks that have been the target of research worldwide. Given this scenario, these authors concluded that AMPs

have promising applications in the clinical setting, but they highlighted that their properties and modes of action still require further studies. Together, all the results regarding the MIC values of melittin against cariogenic bacteria presented in this work agree with the properties reported for this peptide.

## CONCLUSION

In conclusion, we have found a substance with potential use in new bactericidal tests. Indeed, melittin proved to be active against the bacteria present in the oral cavity. Our results on apitoxin cannot be compared with other literature works, due to the lack of specific studies on the use of apitoxin against oral pathogens. New studies based on a chemical, pharmacological, and clinical approach must be conducted, because apitoxin and one of its main components, melittin, have been shown to be highly effective against oral pathogens.

## RESUMO

O presente estudo avaliou pela técnica da Concentração Inibitória Mínima (CIM) o potencial antibacteriano da apitoxina produzido pela abelha *Apis mellifera* frente aos microrganismos causadores da cárie dental. Apitoxina foi avaliada na forma *in natura* e comercial. A ação antibacteriana dos principais componentes da apitoxina, fosfolipase A<sub>2</sub> e melitina foram também avaliados, isolados e em combinação. As bactérias testadas foram: *Streptococcus salivarius*, *S. sobrinus*, *S. mutans*, *S. mitis*, *S. sanguinis*, *Lactobacillus casei* e *Enterococcus faecalis*. Os resultados da CIM da apitoxina comercial e *in natura* foram muito próximos, variando entre 20 µg/mL e 40 µg/mL, ambas apresentaram uma boa atividade antibacteriana. Entre os componentes, a melitina foi a mais ativa, exibindo valores de CIM muito promissores, variando de 4 a 40 µg/mL. A fosfolipase A<sub>2</sub> apresentou resultados maiores que 400 µg/mL. A associação entre a melitina e fosfolipase A<sub>2</sub> apresentou resultados da CIM que variaram entre 6 µg/mL e 80 µg/mL. Considerando que a cárie dental afeta a saúde das pessoas, a apitoxina e seu composto, melitina, possuem potencial aplicação frente aos patógenos bucais.



**Palavras-chave:** atividade antibacteriana, apitoxina, Melitina, Fosfolipase A<sub>2</sub>, cárie dental, patógenos bucais.

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