Psammaplin A: A Putative Adjuvant for DNA Damaging Therapies

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Abstract

Psammaplin A is a phenolic marine metabolite that exhibits antitumor properties. There is evidence that Psammaplin A is a DNA methyltransferase (DNMT) inhibitor that sensitizes human cancer cells by suppressing DNA repair activity. There is also evidence that the drug is a histone deacetylase. The aim of this experiment is to test the efficacy of Psammaplin A as an anticancer therapeutic agent by comparing its effect on lung cancer cells NCI-H226 Bap1 null cells and on human neuroblastomal SKN cells. In the experiment SKN cells are used because they are more sensitive than normal cells, and toxicity can be discerned better with the use of SKN cells. Results show that at a concentration between 1/10 000 μL and 1/1000 μL of Psammaplin A significantly inhibits cell growth of the Bap1 null cells while presenting minimal toxicity to SKN cells. The results were particularly strong when CPT (a DNA-damage inducing drug) was added to the cells. This indicates that Psammaplin A is a potential adjuvant for cancer patients, particularly for Bap-1 null lung cancer patients being treated with DNA-damage inducing therapies. Results also confirm that Psammaplin A does not affect neuroblastoma or neuroblastomal pathways. Results also indicate, since Psammaplin A inhibition differs from MG132 inhibition of cell proliferation, that Psammaplin A does not inhibit proteasomes.

Introduction

Psammaplin A inhibits cancerous growth

Psammaplin A is a marine metabolite found in sea sponges. It is a phenolic compound and a neutral organic compound [1]. Psammaplin A is also a dimer [2]. Because it is a neutral phenolic compound it will not react with the hydrochloric acid in the gastric chyme and will therefore pass unchanged into the gastrointestinal tract, where it is absorbed. This is significant because certain drugs are broken down in the stomach, and so the hormone must be injected. An example of this is growth hormone, which is prescribed to girls with Turners syndrome. The same goes for insulin, an important hormone for diabetics that is injected. Both of these hormones are proteins, which are readily degraded into smaller peptides or amino acids by the stomach acid and the gastric enzyme pepsin. Psammaplin A, because it is a neutral organic compound, would not react with the gastric chyme solution of the stomach, and because it is not a polysaccharide will not be broken down by salivary amylases. As for bile salts and other lipases presented in the gastrointestinal tract, further testing to verify how Psammaplin A reacts in that environment will determine if Psammaplin A is ingestible or not.

Psammaplin A is a drug that exhibits antitumor and anticancer properties, as well as varying effects in several different pathways. The drug shows antitumor properties via angiogenesis inhibition *in vitro* [1] (Figure 1a). Psammaplin A inhibits amino peptidase N (APN). APN is a key factor in tumor cell invasion and angiogenesis. Thus, by inhibiting APN, Psammaplin A thereby inhibits angiogenesis. There is also evidence that Psammaplin A inhibits leukemia cells [2]. Also, Psammaplin A exerts epigenetic effects through histone deacetylation [3], arresting the cell cycle and inducing apoptosis in human endometrial cancer cells in a dose-dependent manner. More specifically, HDAC1 appears to be a key histone deacetylase that Psammaplin A affects. Further, Kim et al. found that Psammaplin A increased radio sensitivity in cell lines, and that this effect is evidently due to inhibition of DNA repair [4]. Psammaplin A was found to significantly increase radiation killing in lung cancer and glioblastomal

cell lines [4]. According to Kim et al., a Western blot analysis revealed a drastic depletion of DNA methyltransferases (DNMTs) by Psammaplin A, specifically DNMT1 and DNMT3A. This link to DNMTs provides a bridge between Psammaplin A and PcGs [4].

PcGS and DNA repair mechanisms

Polycomb group proteins (PcGs) are well characterized protein complexes that are implicated in epigenetic regulation of gene expression [5]. PcGs play a key role in defining cell phenotype. PcGs are also necessary for proper embryonic development and specification of stem cell gene expression profiles, controlling self-renewal, pluripotency and differentiation. There are two types of polycomb repressive complexes (PRCs) that are particularly significant: PRC1 and PRC2. PRCs exhibit silencing functions through direct alteration of chromatin conformation (PRC1 ubiquitylation) or by recruitment of DNA methyltransferases (PRC-2). According to Gieni et al., PcG
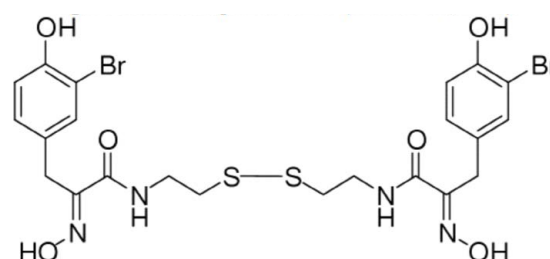

Figure 1a: Psammaplin A dimer [1].

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overexpression in dysplastic tissue appears to be an early mechanism in oncogenesis through which significant tumor suppressor loci become attenuated [5]. The implication of PcG overexpression in cancer cell phenotype and in disease progression and metastasis means that Psammaplin A's DNA methyltransferase (DNMT) inhibiting properties has significant implications on cancer therapy.

HDAC1, a histone deacetylase, negatively regulates gene expression [6]. The p65 (RelA) subunit of NF-kB interacts with the HDAC1 and HDAC2 cofactors to inhibit gene expression. NF-kB plays a significant role in signal transduction in the cell, which affects gene expression. Moreover, there is also evidence that HDAC1 complex with DNMT1, Rb, and E2F1 to repress transcription from E2F-responsive promoters [7]. The results establish a link between DNA methylation, histone deacetylase, DNA sequence-specific DNA binding activity, and a growth-regulatory pathway that is disrupted in nearly all cancer cells [7]. In addition, this interlinking of so many factors explains Psammaplin A's ability to inhibit so many cancer cell types.

Aim and Clinical Relevance

The aim of this experiment is to test the efficacy of Psammaplin A in cancer treatment. Psammaplin A is a significant drug because of its antitumor properties; Psammaplin A has been shown to inhibit several types of cancerous cell lines. In vitro, Psammaplin A inhibits lung cancer and glioblastomal cell lines [4], human endometrial cancer cells [3], and leukemia cells [2]. Psammaplin A's capability to inhibit so many cancer cell types establishes Psammaplin A's potential as a broad-spectrum anticancer drug. In this experiment, NCI-H226 Bap1 null lung cancer cells and the control human neuroblastomal SKN cells are treated with varying concentrations of Psammaplin A or MG132. SKN cells are used due to its high sensitivity to toxicity, and to confirm Psammaplin A does not inhibit the SKN neuroblastomal cells. In this way the SKN cell line may be used as an indicator of Psammaplin A's toxicity.

MG132 is a drug used as a baseline for comparison; looking at a drug already known to inhibit lung cancer cells, Psammaplin A's effectiveness can then be compared to an existing drug (MG132) that also inhibits lung cancer cells. Bang's paper [8] illustrates MG132's lung cancer inhibiting properties. MG132, however, does not seem to affect the Bap1 pathway; MG132 inhibits growth of a different cancer cell line. MG132 is a proteasome inhibitor [9]; comparing Psammaplin A and MG132 inhibition of cell proliferation would indicate if Psammaplin A is also a proteasome inhibitor. Psammaplin A's cancer cell eradication properties and toxicity to normal cells will be measured and compared to those of the drug MG132. Cells will be treated with varying concentrations of either drug as well as Cisplatin (CPT). CPT is a DNA damage-inducing drug, and for all intents and purposes is equivalent to ionizing radiation. CPT is used to determine how well cells recover from CPT induced insults to DNA when treated with Psammaplin A or MG132.

Methods

Reagent list

Psammaplin A (Sigma-Aldrich, product no. P8749-5MG), MG132 (Sigma-Aldrich, product no. M7449).

Experiment

NCI-H226 Bap1 null lung cancer cells and SKN cells were used in this experiment. The NCI-H226 cells were grown in RPMI 1640 medium with 10% fetal bovine serum, and the SKN cells were grown in DMEM with 10% fetal bovine serum. Cells were incubated at 37°C and 5% CO_2. Cells were seeded into μ-Slide 8-well uncoated sterilized Ibidi lab ware plates and incubated for 42 hours in ~1.5 mL of media. The cells are then treated with drug (Psammaplin A or MG132) in varying concentrations: 0, 1/10 000, 1/1000, and 1/500 μL. This setup was duplicated, but in the duplicate setup the cells were also in 1 μM CPT. Once cells are treated with drug, cells are incubated for another hour, then stained with crystal violet and counted under a basic light microscope.

The drug Psammaplin A was tested on NCI-H226 BAP1 null lung cancer cells and on SKN cells. Cells were seeded and incubated for 42 hours, and then the media was replaced with fresh media. Cells were then treated with varying amounts of Psammaplin A or MG132 to achieve an end concentration of drug in the well of 1/10 000 μL, 1/1000 μL, or 1/500 μL. A duplicate set of cells of each line were then treated with CPT to induce double-stranded breaks, and varying concentrations of either Psammaplin A or MG132. Cells were then incubated one hour, then stained with crystal violet and counted under microscope. The number of cells in the microscopic field of view were counted and recorded.

Results

When the cells are treated with both CPT and Psammaplin A, a similar pattern is apparent, but the overall cell number in the field of view of the microscope was considerably lower. With only CPT, the cell number count was 240 cells. With the additional presence of Psammaplin A at concentrations of 1/10 000 μL or 1/1000 μL, the cell number declines dramatically to 15. For a Psammaplin A concentration of 1/500 μL, however, the cell count was 50 (Table 1).

Simple linear regression results I

Dependent variable: cpt

Independent variable: drug

cpt = 230.15053 - 114065.195 drug

Sample size: 4

R (correlation coefficient) = -0.9888

R-sq = 0.97778153

Estimate of error standard deviation: 19.632994 (Tables 2 and 3).

Psammaplin A or MG132 Concentration(μL)	Treatment Psammaplin A		Treatment MG132	
	1 μM CPT	no CPT	1 μM CPT	no CPT
NCI-H226 BAP1 null cells (#cells per field of view)				
0	80	240	250	300
1/10 000	15	150	200	250
1/1000	15	10	112	65
1/500	50	200	5	7
SKN cells (# cells per field of view)				
0	800 000	900 000	10 000	200
1/10 000	800 000	900 000	200	150
1/1000	800 000	900 000	800 000	80
1/500	600 000	800 000	800 000	500 000

Source : Jamile Charkie, Cross-Cancer Institute

Table 1: Two cell lines were tested with varying concentrations of Psammaplin A or MG132, in combination with 1 μM CPT or no CPT.

Parameter	Estimate	Std. Err.	DF	95% L. Limit	95% U. Limit
Intercept	230.15053	13.607027	2	171.60422	288.69684
Slope	-114065.195	12158.343	2	-166378.33	-61752.07

Table 2: Parameter estimates.

Source	DF	SS	MS	F-stat	P-value
Model	1	33925.84	33925.84	88.015175	0.0112
Error	2	770.90894	385.45447		
Total	3	34696.75			

Table 3: Analysis of variance table for regression model.

Simple linear regression results II

Dependent Variable: no cpt

Independent Variable: drug

no cpt = 267.86386 - 144985.62 drug

Sample size: 4

R (correlation coefficient) = -0.9554

R-sq = 0.91272396

Estimate of error standard deviation: 51.191734 (Tables 4 and 5).

It is evident from Figure 1b that MG132 inhibits cell growth of the BAP 1 null cells. Figure 2 depicts a negative correlation between cell growth and the concentration of the drug MG132. Upon statistical analysis, a simple linear regression between the amount of MG132 and number of cells in presence of CPT gave an R-value of -0.9888 with a 95% confidence interval (see Simple Linear Regression Results I). This R-value indicates a very strong negative correlation between the amount of MG132 and cell count in the presence of CPT. The simple linear regression between MG132 and cell count in absence of CPT presented an R-value of -0.9554 (see Simple Linear Regression Results II). These two R-values suggest a high correlation between the amount of drug MG132 and the number of viable cells, regardless of CPT, although in presence of CPT the correlation appears slightly stronger.

The drug MG132 appears to aid cell growth of SKN cells (Figure 2). The graph presents a significant increase in cell number as MG132 concentration increases. In presence of CPT, the cell number jumps from below 100 000 at MG132 concentrations of 0 and 1/10 000 µL to a cell count of 800 000 at an MG132 concentration of 1/1000 and 1/500 µL. Without CPT, a jump us observed at an MG132 concentration above 1/1000. The cell count below 100 000 jumps to 500 000 in a MG132 concentration of 1/500 µL.

Discussion

Efficacy of Psammaplin A

From the aforementioned data it is evident that the drug Psammaplin A is a potent anti-cancer drug. In particular, Psammaplin A exhibits potential as an adjuvant complementary to DNA damage inducing therapies. Looking at Figure 1, lung cancer cell plated wells treated with Psammaplin A did show inhibition of cell growth. Cell number decreased heavily with a 1/10 000 µL concentration of Psammaplin A. Cell number decreased further when cells were treated with a 1/10 000 µL concentration of Psammaplin A. The cell number for a treatment of Psammaplin A at a concentration of 1/500, however, exhibited less killing than the previous concentrations of Psammaplin A, but still more killing than the control cells not treated with Psammaplin A. It is also significant that when cells were treated

with a Psammaplin A concentration of 1/500 µL with CPT, the same phenomenon was observed. Irrespective to the presence of CPT, a Psammaplin A concentration of 1/500 µL exhibited more cell growth than for a Psammaplin A concentration of 1/1000 µL, but still less growth was present in absence of Psammaplin A. Further testing would need to be done to better understand this phenomenon, but it is highly likely that, at concentrations above 1/1000 µL, Psammaplin A triggers an afflux pump or the expression of some silent gene(s) in the cells that inhibit this drug. Nevertheless, Figure 3 shows that, at a concentration of 1/1000 µL, Psammaplin A exhibits potent antitumor capabilities against the Bap 1 null lung cancer cells.
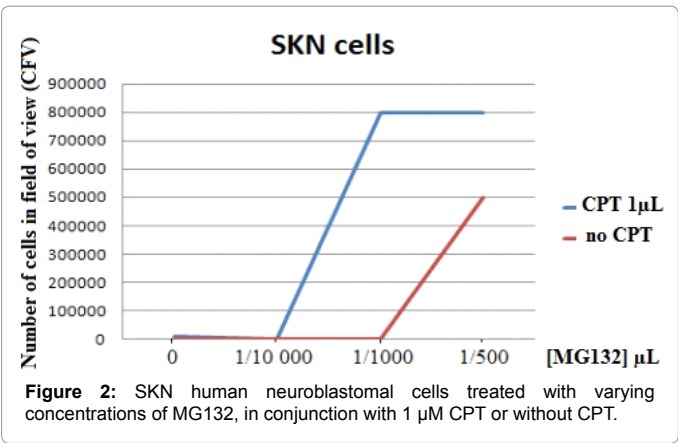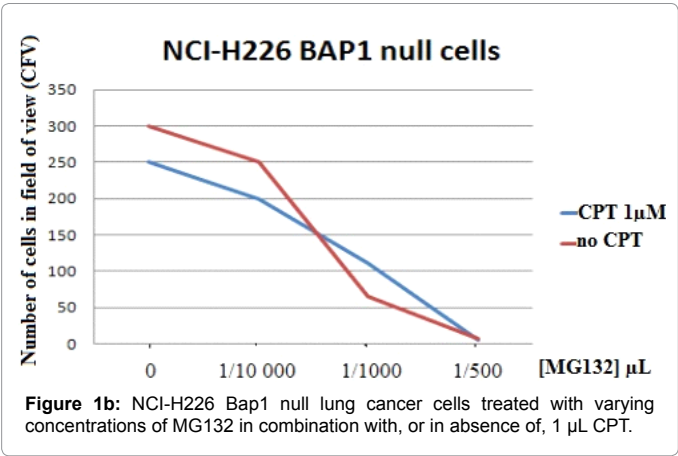
Psammaplin A as an adjuvant

Comparing the two linear functions of Figure 1a, it is evident that inhibition of the lung cancer NCI-H226 Bap1 null cells by Psammaplin A was considerably stronger, and more constant, for all concentrations when cells were also treated with CPT. The blue line representing
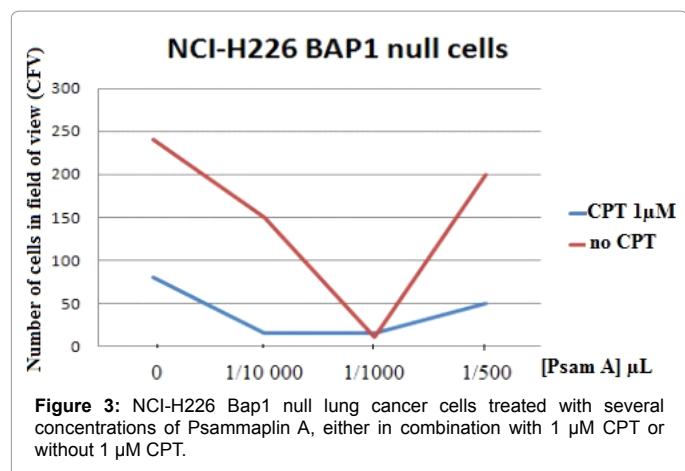
Parameter	Estimate	Std. Err.	DF	95% L. Limit	95% U. Limit
Intercept	267.86386	35.479424	2	115.20822	420.5195
Slope	-144985.62	31702.074	2	-281388.62	-8582.605

Table 4: Parameter estimates.

Source	DF	SS	MS	F-stat	P-value
Model	1	54811.812	54811.812	20.915802	0.0446
Error	2	5241.187	2620.5935		
Total	3	60053			

Table 5: Analysis of variance table for regression model.

Figure 1b: NCI-H226 Bap1 null lung cancer cells treated with varying concentrations of MG132 in combination with, or in absence of, 1 µL CPT.

Figure 2: SKN human neuroblastomal cells treated with varying concentrations of MG132, in conjunction with 1 µM CPT or without CPT.

Figure 3: NCI-H226 Bap1 null lung cancer cells treated with several concentrations of Psammaplin A, either in combination with 1 μM CPT or without 1 μM CPT.

Psammaplin A and CPT treated cells is much lower and flatter than the red line representing cells not treated with CPT (Figure 3). With no added Psammaplin A, the Bap 1 cell count was 240, but with CPT the cell count was 80 (Table 1). At a Psammaplin A concentration of 1/10 000 μL, the cell count was 150, and with CPT, 15. At a drug concentration of 1/500 μL, cell count was 200, and with CPT the cell count was 50. The exception was when the cell count was 10 and with CPT 15 for a Psammaplin A concentration of 1/1000 μL. Nevertheless, the fact that Psammaplin A consistently presented much stronger inhibition when combined with CPT suggests Psammaplin A may be better as an adjuvant complementing DNA damage inducing therapy rather than a primary therapeutic agent.

Psammaplin A presented minimal toxicity to SKN cells (Figure 4). Cell numbers were very high, dipping at a Psammaplin A concentration of 1/500 μL. The cells showed the same trend whether or not they were treated with CPT, however, cell numbers were higher for cells not treated with CPT (Figure 2). From the graphs (Figures 3 and 4), the ideal parameters for cancer therapy would be a Psammaplin A concentration between 1/10 000 and 1/1000 μL, depending on individual circumstances and side-effects presented. Further testing of Psammaplin A *in-vivo*, such as in a mouse, would show how the drug Psammaplin A presents long-term in an organism.
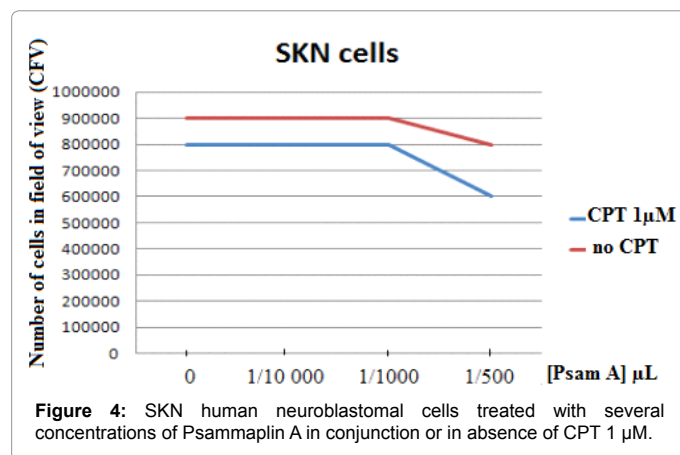
Psammaplin A appears to be a better drug than MG132

Comparing Psammaplin A to MG132, it is apparent that Psammaplin A is a better drug. Bap 1 null cell count in presence of MG132 (Figure 1) gives very good r-values (Simple Linear Regression Results I and II). The r-values -0.988 (with CPT) and -0.9554 (without CPT) indicate a very strong negative corellation between cell growth and the concentration of CPT. Despite this, Psammaplin A still shows better inhibition of the Bap 1 null cells below a concentration of 1/500 μL. With respect to Bap 1 cells, we see that cells when combined with CPT, Psammaplin A exhibits even more killing of NCI-H226 Bap1 null cells at lower concentrations than MG132 with CPT (Figures 1 and 3). In absence of CPT, both drugs show very similar inhibition of the lung cancer cells. Bap 1 null cell count in [1/10 000] Psammaplin A was around 150 and around 15 with CPT (Table 1). By comparison, for the same concentration of MG123, the cell count was around 250 and 200 with CPT. At [1/1000] μL of Psammaplin A, cell count was 10 and 15 with CPT, while for [1/1000] μL of MG132 the cell count was 65 and 112 with CPT. Because Psammaplin A and MG132 affect cell proliferation so differently, it seems Psammaplin A does not inhibit proteasomes as MG132 does.

Examination and analysis of SKN cell growth in Psammaplin A or MG132 further illustrate Psammaplin A's superiority to MG132. In Figure 4 we see no inhibition of the SKN cells, except at a Psammaplin A concentration of 1/500 μL, indicating the low toxicity of Psammaplin A. This also indicates that Psammaplin A would not be ideal for treatment of neuroblastomal patients. In the presence of CPT, we also see a steady cell count when Psammaplin A is added in concentrations below 1/500, but the cell counts are slightly below the respective cell counts without CPT. Again, this indicates that Psammaplin A toxicity is low. MG132, however, actually presented an increase in SKN cell growth. CPT treated SKN cells presented a cell count of 10 000, dips with addition of MG132, but then cell count rises to 800 000 (Table 1). In Figure 2, the SKN cell count was initially below 100,000. At [1/10,000] μL MG132, the cell count is still below 100 000. However, at [1/1000] μL MG132, the cell count jumps to 800,000, and then the graph plateaus. In CPT, the cell count jumps at [1/500] μL MG132. This indicates that in SKN cells a high enough concentration of MG132 induces efflux pumps or silenced resistance genes, or the drug does not affect SKN cells.

Conclusion

Psammaplin A presents a great deal of potential, particularly as an adjuvant to complement DNA damage inducing therapies, such as ionizing radiation. As mentioned previously, Psammaplin A inhibits lung cancer and glioblastomal cell lines [4], human endometrial cancer cells [3], and leukemia cells [2] *in vitro*. From this paper's aforementioned experiments it is evident that Psammaplin A presents toxicity to BAP1 null lung cancer cells, particularly in the presence of the DSB inducing drug CPT. A repeat experiment with radiated NCI-H226 Bap1 null cells and SKN cells rather than CPT-treated cells should present similar results, thereby confirming that Psammaplin A would likely make a good adjuvant. Further, Psammaplin A shows very little toxicity to normal SKN cells, even with CPT. Because Psammaplin A does not inhibit SKN neuroblastomal cells, it is clear that Psammaplin A is not an ideal treatment for neuroblastomal patients. These conclusions could be strengthened with MTT or MTA assays, although the results obtained from this experiment make its point. Further testing to verify the exact mechanisms that result in Psammaplin A's inhibition may be beneficial, although Psammaplin A has already been shown to affect many other pathways already. Having said this, it is evident from the proliferation assays of this paper that Psammaplin A does not inhibit neuroblastomal pathways or proteasomes, since Psammaplin A does not inhibit proliferation of SKN cells, and because

Figure 4: SKN human neuroblastomal cells treated with several concentrations of Psammaplin A in conjunction or in absence of CPT 1 μM.

the proteasome inhibitor MG132 does not affect proliferation the same way Psammaplin A does.

Psammaplin A is a potent anticancer drug that deserves further attention. Future testing in normal cells and animal models such as mice would show how Psammaplin A affects the organism and would give an idea of the side-effects and long-term effects to be expected. Also significant would be experiments combining Psammaplin A and other known DNA damage inducing therapies to test which combinations work best.

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