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Developing a Nanobody to Target the Interleukin-31 Receptor as a Treatment for Atopic Dermatitis

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ABSTRACT

Atopic Dermatitis (AD), the most common form of eczema, causes chronic pruritus, regularly disrupting sleep, work, and other essential activities. Over 50% of adults with AD have found treatments to be ineffective.

This research seeks to computationally design an effective yet non-invasive treatment for AD for future in-vivo testing. One of the major signaling molecules in the AD pathogenesis pathway is the cytokine interleukin-31 (IL-31).

Treatments for AD targeting the IL-31 receptor exist, however these are monoclonal antibody treatments and require administration via injection. This can be avoided with a smaller therapeutic, like a nanobody, which could be equally effective while having more accessible administration methods.

The structure of the IL-31A subunit was obtained and used in a protein generating software. Multiple different trials specified different binding areas to design nanobodies with both similar, and dissimilar epitopes to nemolizumab, an existing monoclonal antibody treatment. Generation was run in batches of 50, with the top 10 designs based on overall quality being returned for analysis. ipTM scores, design pTM scores, along with buried surface area (BSA) and other measures, were manually calculated and collected. After comparison to the same values in nemolizumab, the overall top design showed similarities suggesting high likelihood of in-vivo success. The ipTM value was below 0.6, so for future studies this design could be improved before in-vivo testing to increase chances of in-vivo success. These results, combined with nanobodies' ease of administration via hydrogel-forming microneedle patches, provide the foundation for a new AD therapeutic.

INTRODUCTION

Atopic dermatitis (AD), the most common form of eczema, is a chronic skin condition that causes dry, itchy, and inflamed skin. It affects roughly 15–20% of children and up to 10% of adults worldwide, making it one of the most common skin diseases globally.[1] People with AD often experience flare-ups that interfere with sleep, school, and daily life, and the condition frequently occurs alongside other allergic diseases like asthma and hay fever.[2] A key driver of the itching and inflammation in AD is a protein called Interleukin-31 (IL-31), which is released by activated CD4+ T helper 2 (Th2), mast cells, and eosinophils during a flare-up.

IL-31 binds to a heterodimeric receptor, with one part being the IL-31 receptor A (IL-3RA), and the other being oncostatin M receptor (OSMR). This receptor is expressed on sensory neurons, macrophages, dendritic cells, basophils, and keratinocytes. Activation of the IL-31RA/OSMR by IL-31 leads to activation of Janus kinase 1 (JAK1)/JAK2, and also STAT3 in non-neuronal cells. In primary sensory neurons, IL-31 triggers Ca^{2+} release, and phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). These signals then ad in activating transient receptor potential (TRP) channels TRPV1 and TRPA1, which leads to depolarization of the neuron, translating the cytokine signal into neuronal firing. The firing of this neuron leads to itching sensations in that area of the body.[3] Because IL-31 plays such a central role in causing these symptoms, blocking the IL-31 receptor is a promising strategy for treating AD.

Current treatments for moderate-to-severe AD include injectable biologic drugs—medicines made from proteins—that target parts of the immune system responsible for inflammation. While these drugs have been effective, they come with significant drawbacks: they are expensive, must be injected into the bloodstream, and can cause unwanted side effects throughout the body because they circulate systemically.[4,5] This project explores a different approach using a nanobody, which is a much smaller type of antibody-like protein originally discovered in camels and llamas. At roughly one-tenth the size of a conventional antibody, nanobodies can reach targets that larger molecules cannot, are cheaper to produce, and are more stable.[6] Rather than being designed through animal experiments, the nanobodies in this study were created entirely using computational protein-design softwares, programs that use artificial intelligence to predict and build protein structures, to design a nanobody that fits precisely onto the IL-31 receptor and blocks it from activating.

Delivering protein-based drugs through the skin is normally very difficult because the outermost layer of skin acts as a barrier that blocks large molecules from passing through. To solve this problem, this project proposes using a microneedle patch—a small bandage-like device covered in microscopic needles, too small to cause pain, that painlessly pierce the outer skin layer and deposit the drug directly where it is needed.[7] This approach would allow the nanobody to be delivered directly to the inflamed skin without requiring an injection or causing widespread effects on the rest of the body. Together, the combination of a computationally designed nanobody targeting IL-31R and a microneedle patch delivery system offers a novel, non-invasive, and targeted approach to treating atopic dermatitis—one that could be more accessible, affordable, and comfortable for patients, especially children, compared to existing therapies.

The study aimed to computationally design a nanobody targeting the IL-31 receptor alpha subunit, using nemolizumab's known binding epitope as a structural reference. The target design featured CDR loops of 11,17, and 15 amino acids for CDR1, CDR2, and CDR3 respectively, with a common amino acid framework. These dimensions were carefully selected to maximize interface coverage while maintaining the compact nanobody scaffold. Designs were evaluated against nemolizumab based on predicted binding affinity, among other values, including suitability for microneedle patch delivery.

DESIGN METHODOLOGY

The plan was to create initial nanobody designs using an open-source protein-generating software called Boltzgen[8], which was accessed using a free version of the webserver Rowan Science. It took 17 trial runs with various input information to determine what the limitations of the software were, and what input requirements it would take to return the most accurate and desirable results

For the final runs, the following information was input into the Boltzgen software: In the "Protein Sequences" section, the chain ID was labelled "N", and the sequence that was inputted was:

QVQLVESGGGLVQPGGSLRLSCAAS11FRQAPGKGREFVS17RFTISRDNAKNTVYLQMNS

LKPEDTAVYYC15WGQGTLVTVSS

This sequence was used because it contained the standard amino acid sequence for the non-variable sections of a nanobody. The numbers "11", "17", and "15" within that sequence represent the 3 CDR loops within the nanobody that the nanobody could generate as any amino acid combination.

The PDB ID for the "Interleukin-31 Receptor D1D2 in complex with Nemolizumab derived scFv", 9EZA, was input into the site so that it could import the PDB file for that structure. All Chains except chain A were removed, and the file was refined by the site.

The protocol was always set to "Nanobody-Anything: design single-domain antibodies". For trial runs to understand the software, the number of designs was set to 10, and the budget was set to 2. For the actual runs which created the designs later analyzed, the number of designs was set to 50, and the budget was set to 10. The runtime limit was set to match the number of credits there were remaining in the Rowan Science account to prevent the run from running out of credits before coming to completion.

5 runs of nanobody generation were done, for a total of 250 designs being generated and 50 being analyzed during the duration of the research. The only input information that differed based on each run was the specified binding and non-binding regions for the software. Every time that any specifications were given for binding regions, the Boltzgen software returned no results, so only non-binding regions were used to guide generation. The different input values were:

Trial 1: No Restrictions

Trial 2: No Restrictions

Trial 3: Restricted residues 155-261

Trial 4: Restricted residues 220-261

Trial 5: Restricted residues 200-261

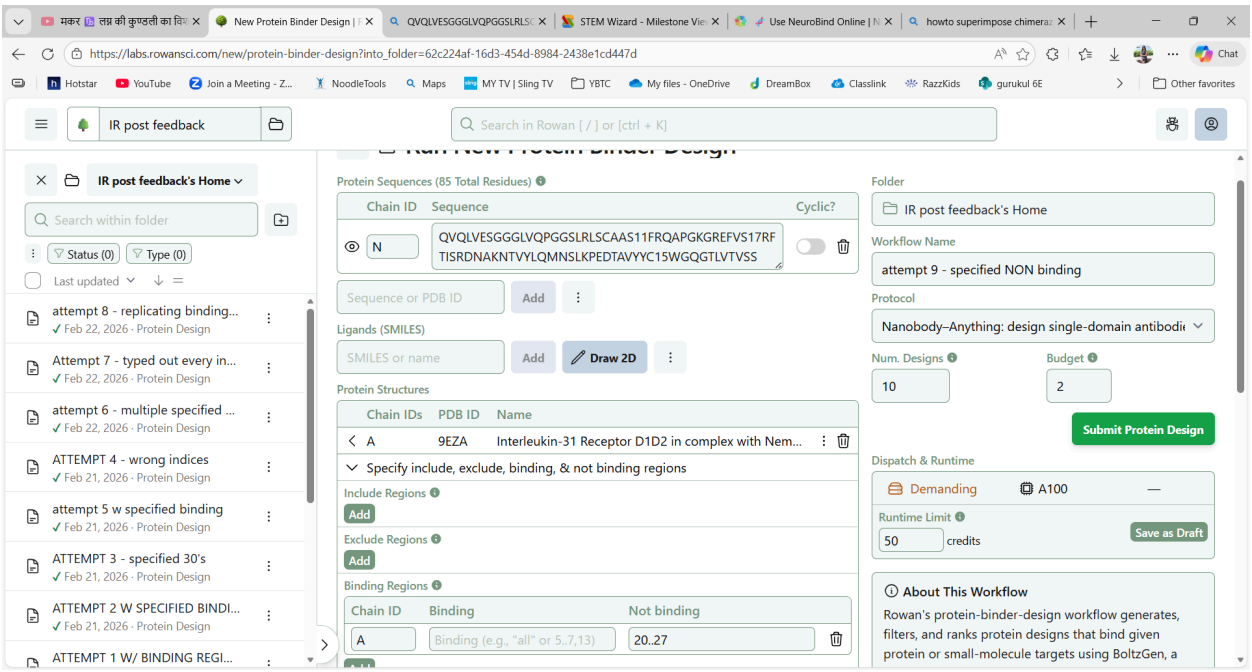


Figure 1- Image showing the input information for nanobody generation within BoltzGen

These input values for epitope restrictions were determined based on two reasons: The binding epitope of Nemolizumab, and the binding epitopes of earlier runs. After runs 1 and 2, some designs were analyzed to determine which residues were most commonly binded to. These were then strategically restricted in runs 3-5 to increase epitope diversity, and attempt to create some designs with epitopes comparable to that of Nemolizumab.

TESTING METHODOLOGY

Originally, the plan was to analyze the nanobodies by superimposing the nanobody-receptor complexes over the Nemolizumab-receptor complexes, and using Jaccard's index[9] to calculate the level of epitope overlap. After designs were generated in the first stage of this research project, some were superimposed and it was determined that there was very little or no epitope overlap, so the following is the way that the nanobodies were analyzed as an alternative.

After the nanobodies were all generated, they were measured across 6 categories: iPTM, Design PTM, buried surface area, ΔG, K_d, and aggregation propensity.

iPTM and Design PTM values are both measures of confidence that were returned by the Boltzgen software after generation[8]. iPTM is the measure of confidence in the epitope of the design, or how confident it is that location on the target that the software predicted is accurate to real life. Design PTM is a measure of confidence in the folding and structure of the nanobody design itself[10,11].

Buried surface area values were determined using the structural visualization tool ChimeraX[12]. The PDB files for the nanobody-IL-31a complexes were downloaded from the results of the 5 runs, and then uploaded into ChimeraX. From here, the command "measure BuriedArea" was used once per complex, and the A (receptor) and B (nanobody) chains were selected. This command returns 3 values-the surface area for each individual chain, and the buried surface area between the two- but only the buried area was kept for analysis.

Then, the designs' binding affinity was analyzed by inputting the PDB files into the tool PRODIGY[13], which returned both ΔG and K_d values. For both values, the smaller the number, the stronger the binding affinity between the nanobody and the receptor. A more negative deltaG implies a strong affinity, and a tiny dissociation constant means a low rate of detachment, so the drug will be longer-lasting.

The tool Aggrescan3D[14] was used to analyze the aggregation propensity for all of the amino acids in the nanobodies, which was added up for a total aggregation propensity because all of the nanobodies designed in this project have the same number of amino acids. The more negative this score, the less likely the nanobody is to aggregate, or clump together, which will reduce aggregation-related manufacturing challenges. Aggregation propensity also serves a measure of solubility, and solubility is incredibly important because the type of microneedle patch that nanobodies are infused into, hydrogel-forming microneedle patches[15], function most efficiently when infused with something highly soluble as it facilitates easy penetration into the skin barrier.

RAW DATA

design name (1.1 - run 1, first design based on quality score	iPTM	Design pTM	Buried Surface Area	DeltaG (kcal mol^-1)	Kd (mol) at 37C	A3D score
Nemolizumab	N/A	N/A	809.56	-10.6	3.40E-08	-145.755
1.1	0.327	0.785	815.94	-10.7	2.70E-08	-67.2402
1.2	0.282	0.785	616.23	-8.9	5.10E-07	-74.5929
1.3	0.256	0.796	853.69	-13.1	6.00E-10	-63.7191
1.4	0.251	0.788	804.36	-12.2	2.60E-09	-67.5616
1.5	0.326	0.783	795.52	-11.4	9.40E-09	-80.4607
1.6	0.247	0.783	1146.5	-13.5	3.10E-10	-78.6851
1.7	0.293	0.78	841.52	-11.6	6.50E-09	-79.1195

1.8	0.231	0.788	451.1	-9.4	2.30E-07	-65.6589
1.9	0.229	0.788	526.94	-10.5	3.90E-08	-61.4765
1.10.	0.374	0.78	473.08	-10.4	4.60E-08	-61.6917
2.1	0.333	0.791	949.63	-15.1	2.40E-11	-69.1131
2.2	0.39	0.79	741.23	-10.3	5.40E-08	-85.3983
2.3	0.398	0.787	651.68	-8.6	8.00E-07	-67.2013
2.4	0.299	0.79	598.26	-11.4	9.60E-09	-83.5285
2.5	0.29	0.79	767.28	-10.9	2.20E-08	-82.1399
2.6	0.291	0.798	512.89	-9.4	2.30E-07	-76.4697
2.7	0.237	0.796	635.59	-10.8	2.40E-08	-73.3936
2.8	0.445	0.776	622.79	-9	4.20E-07	-68.4621
2.9	0.235	0.778	520.77	-10.6	3.40E-08	-81.0466
2.10.	0.247	0.784	417.65	-8.2	1.70E-06	-75.6957
3.1	0.176	0.804	826.31	-11.2	1.20E-08	-73.0686
3.2	0.23	0.786	1066.9	-13.7	2.30E-10	-79.1173
3.3	0.244	0.801	673.54	-9.4	2.40E-07	-77.2731
3.4	0.195	0.786	888.79	-11.9	4.10E-09	-59.1246
3.5	0.151	0.782	948.31	-14.3	8.70E-11	-67.2897
3.6	0.182	0.795	482.51	-8.6	9.30E-07	-60.1817
3.7	0.166	0.782	972.59	-12.9	8.30E-10	-71.8979
3.8	0.151	0.786	760.38	-10.8	2.40E-08	-69.9601
3.9	0.227	0.781	785.22	-10.5	3.70E-08	-63.1169

3.10.	0.152	0.781	729.39	-11.3	1.00E-08	-71.4764
4.1	0.65	0.779	938.77	-11.2	1.30E-08	-68.1961
4.2	0.644	0.779	737.25	-10.7	2.70E-08	-69.2344
4.3	0.359	0.784	565.61	-9.7	1.50E-07	-73.5971
4.4	0.374	0.777	773.26	-11.7	5.70E-09	-74.9494
4.5	0.596	0.777	679.89	-9.1	4.10E-07	-77.3284
4.6	0.291	0.778	648.55	-9.1	3.60E-07	-78.5698
4.7	0.353	0.774	909.06	-17.2	7.40E-13	-67.8996
4.8	0.24	0.784	598.37	-10.5	3.70E-08	-78.906
4.9	0.206	0.787	854.28	-12.7	1.10E-09	-85.5098
4.10.	0.213	0.789	649.49	-9	4.70E-07	-83.3659
5.1	0.36	0.786	748.59	-10.4	4.90E-08	-70.3002
5.2	0.376	0.783	855.24	-12.1	3.10E-09	-76.0992
5.3	0.428	0.781	710.43	-9	4.30E-07	-88.1963
5.4	0.251	0.787	689.8	-10.5	4.20E-08	-71.5125
5.5	0.334	0.787	615.62	-10.6	3.30E-08	-77.2944
5.6	0.187	0.781	950.98	-14	1.50E-10	-85.1111
5.7	0.271	0.795	584.84	-10.8	2.60E-08	-74.0391
5.8	0.3	0.78	565.84	-9.7	1.50E-07	-65.9388
5.9	0.213	0.779	719.89	-12.9	8.40E-10	-85.3119
5.10.	0.197	0.78	762.64	-11	1.90E-08	-72.6454

Figure 2 - Table containing the raw data for all designs, and Nemolizumab. Top designs were 1.6, 5.6, and 3.2

DATA ANALYSIS

To use all of the different measurements when comparing the nanobody designs, they needed to be converted to unitless measurements that would allow for a fair comparison. A min-max normalization was applied to all of the data points to convert them to a value between 0 and 1. A different formula was used depending on whether it was better for that particular value to be as small or as large as possible. A score of 0 meant a data point in that category was the absolute worst compared to all of the other nanobody designs, and a score of 1 meant it was the absolute best.

Data where larger values are better:

$$\frac{X - Xmin}{Xmax - Xmin}$$

Data where smaller values are better:

$$\frac{Xmin - X}{Xmax - Xmin}$$

Figure 3 - Min-max normalization equations that were used during data analysis

After this, the different values were weighted differently based on their importance. iPTM and Design PTM were weighted at 7.5% each, as they reflecting computational prediction certainty, which give confidence an overall weighting of 15%. Buried Surface Area, ΔG, and Kd were all weighted at 20% because they all quantify the strength the stability of the nanobody-receptor interaction, and they indicate potential in-vivo inhibitory efficacy. The A3D score received the highest individual weighting at 25% because solubility is a prerequisite for effective administration, and binding affinity is worthless if the therapeutic can't be administered using the target method. After applying this weighting formula (figure 4), the designs with the highest score returned were the designs with the highest likelihood of success and should be tested further.

$$(iPTM) * 0.075 + (Design\ PTM) * 0.075 + (BSA) * 0.2 + (Kd) * 0.2 + (A3D) * 0.25$$

Figure 4 - Overall formula used to apply weighting to the raw data

RESULTS

The top 3 designs, which were determined through scores after data analysis, were designs 1.6 (the 6th design from the 1st run), 5.6, and 3.2, in that order. These top designs all have buried surface area, ΔG, Kd, and A3D values that suggest high inhibitory success. These values were all comparable to the values of Nemolizumab, determined through the same methods, except for the A3D value, but this discrepancy is

due to the fact that Nemolizumab has many more amino acids due to the fact that it's a full-length monoclonal antibody, and Aggrescan3D determines total A3D score by adding up the scores for every amino acid, so this is not expected to affect the comparison.

Design Name	iPTM	Design PTM	BSA	ΔG	K_d	A3D	Overall Weighting
1.6	0.192385	0.3	1	0.588889	0.999818	0.672836	0.722879
5.6	0.072144	0.233333	0.731742	0.644444	0.999912	0.893876	0.7216
3.2	0.158317	0.4	0.890787	0.611111	0.999865	0.687703	0.714152

Figure 5 - Data table showing the data for the top 3 designs after the min-max normalization and weighting was applied

What is a concern about these top designs, however, is their low iPTM values. Generally, an iPTM value of >0.7 is preferable, because any lower indicates low confidence in the location on the target where the nanobody will bind to. The top 3 designs have iPTM values of 0.247, 0.187, and 0.23 respectively. This indicates there is a likelihood that, when the nanobody is synthesized in real life, the folding will be accurate to the way that was predicted, but it may bind to another location on the IL-31a receptor. The target, the alpha subunit of the IL-31 receptor, is far extended from the cell membrane, so there isn't too much of a concern about the nanobody accidentally interacting with the cell membrane, and that affecting the effectiveness. Nevertheless, the low iPTM scores represent a significant limitation that warrants further optimization prior to in-vitro validation.

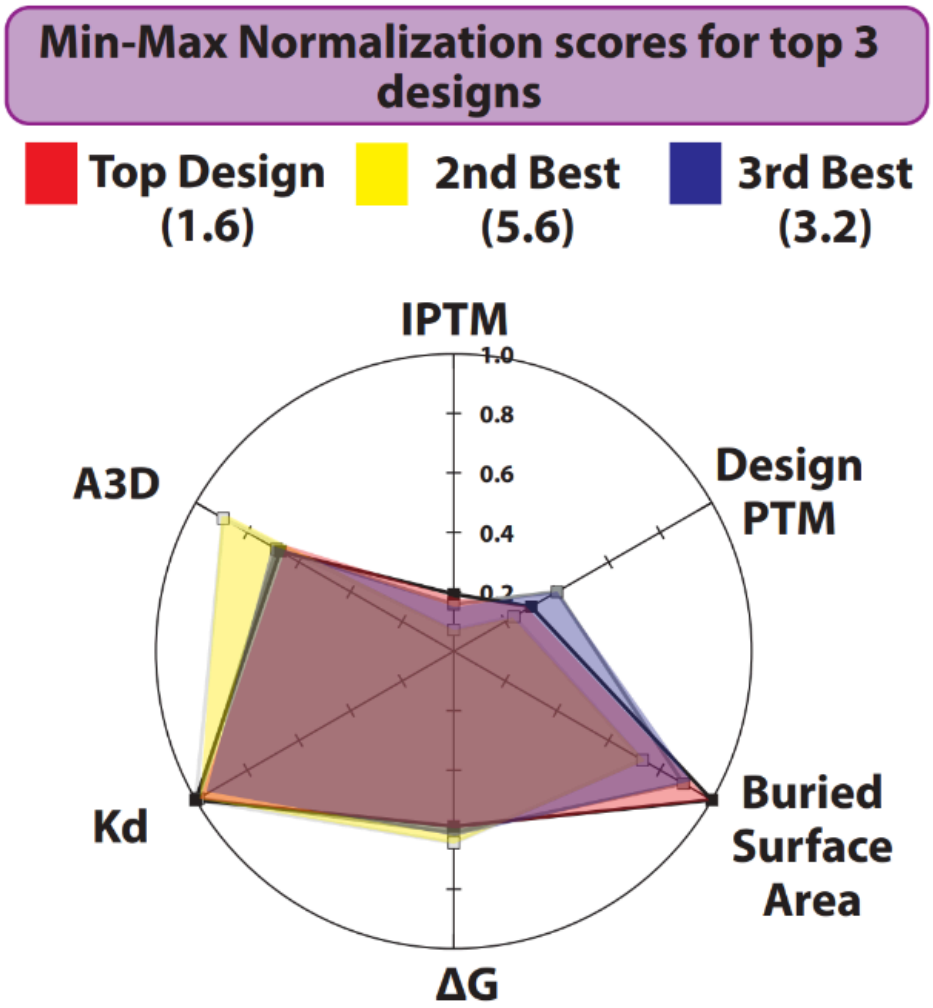


Figure 6 - Radar graph showing how the top 3 designs perform in the 6 data categories, and how they compare to one another.

DISCUSSION

The top 3 designs that were developed through this research project all show significant levels of potential effectiveness at inhibiting the IL-31A receptor, based on the data analysis done. If iPTM values can be optimized, either through additional runs of design generation, or through single residue substitutions based on weak spots in the design folding, then these designs show enough computational effectiveness to be worth the resources it takes to being testing in a laboratory setting.

The top 3 designs all perform similarly in the 6 measurements; however, they have their own noticeable strengths. Design 1.6 had the highest buried surface area out of all 50 of the designs, 5.6 performed significantly better in the A3D score category, and 3.2 performed better in terms of design PTM. This is why all of the top 3 designs are being analyzed in-depth and considered as potential therapeutic treatments, rather than just the top design 1.6.

By looking at figure 2, the similarity between the values of Nemolizumab and the nanobody designs can be seen. The nanobody designs even seem to perform better than Nemolizumab in the ΔG and K_d measurements. Because these were the two primary measures of binding affinity, and Nemolizumab has already been proven to be a functional therapeutic because it's available on the market, this indicates these designs will have similar properties, and therefore similar effects in-vivo.

There are several limitations to this study, especially in terms of sample size. Typical drug discovery studies generate tens of thousands of designs for analyzing before deciding on a final one, but this study could only create 250 due to financial and computational limitations. If access to more credits, or a high-powered GPU became powerful, it would be possible to overcome this limitation. This could theoretically also overcome the issue with the iPTM values, as some design during generation had iPTM values >0.6, showing that it's reasonable, but there were only 2, and they performed poorly in the other tests.

The future steps for this research project involve synthesizing the nanobody through insertion of the gene into a plasmid and then purifying it so that it can be used in an assay. A functional assay could be utilized to determine if application of the nanobody reduces activity in the IL-31 pathway, which would suggest therapeutic effectiveness. If effectiveness is supported, over the course of many years this nanobody can be tested in actual microneedle patches, and eventually become a treatment that can be used in clinical trials.

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