REPORT OF RESEARCH RESULTS

Title: Diagnostic Potential of an LyP-1 Peptide Aptamer for the Detection of Atherosclerotic Plaque

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Summary:
Lyp-1 has previously been reported to bind to p32 protein expressed on atherosclerotic plaques. In this study, we investigated the ability of LyP-1 peptide aptamer to home to the plaques by exploring performance of the LyP-1 peptide aptamer against commercially available anti-p32 antibodies in detecting p32 protein from activated macrophages. Our results demonstrated that the LyP-1 peptide aptamer was capable of binding to p32 protein of both human and animal sources. The small variances and CV% found during data sampling also showcased the consistency in results produced by the LyP-1 aptamer. The contrast between performance test of the LyP-1 aptamer on p32 human standards and cell culture derived p32 highlighted the need to establish LyP-1 optimization based on a separate set of standards. We acknowledged that further optimization with more accurate methods is required to fully discover the potential of the LyP-1 aptamer. Nonetheless, the significant increase in absorbance values evaluated in the selected ELISA assay assessing the p32 protein expression showed promising potential of LyP-1 aptamer utilization as a more effective diagnostic tool.

Aim of Research:

1. To determine if LyP-1 peptide aptamer binds to p32 protein on plaque macrophages
2. To determine if macrophage activation would increase binding of LyP-1 peptide aptamer to p32 protein
3. To determine the efficiency of LyP-1 peptide aptamer binding to p32 protein comparing to the commercially available anti-p32 antibody

Methods of Research and Progression

Several optimization steps were taken to ensure the appropriate utilization of LyP-1 aptamer in this project. Sandwich based ELISA (ELISA) was used as the main evaluation procedure 1) to optimize cell lysate concentration, 2) to optimize LyP-1 concentration and 3) to assess the Lyp-1 binding affinity. Comparisons were performed against human p32 standards. The optimization steps were followed by evaluation of murine RAW264.7 macrophage cell line (RAW cells) and human THP-1 monocyte cell line (THP-1 cells) interaction with either commercial tracer (CT) or LyP-1 aptamer. RAW and THP-1 cells were cultured according to the suppliers protocol (ATCC). Cells were cultures in two groups 1) non-activated and 2) activated. Non-activated cells were cultured in their respective supplier's recommended cell culture media (DMEM and RPMI1640). Activation of RAW cells was performed by the addition of 100µg/mL AcLDL into the cell culture medium for 72 hours. Activation of THP-1 cells was performed by the addition of 100nM PMA into the cell culture medium for 24 hours. ELISA was performed with the commercially available anti-p32 kit (Hycult Biotech gC1qR human ELISA kit, Biomed Diagnostics) to evaluate the p32 binding of the two selected cell lines. Absorbance quantification was performed with a spectrophotometric microplate reader. The absorbance was measured at 450 nm wavelength. The absorbance values (OD450 values) were normalized against total protein concentration. Total protein concentration of all cell lysates of all cell groups was determined by the bicinchoninic assay (BCA Assay Kit, Thermo Scientific). All values were presented as mean values, plus minus the calculated standard deviation. The data analysis was done using student's t-test. Statistical tests were considered significant when $P \leq 0.05$ (noted as one asterisks), $P \leq 0.01$ (noted as two asterisks), $P \leq 0.001$ (noted as three asterisks), and

P≤0.0001 (noted as four asterisks). All statistical analysis was performed using XLSTAT software.

Results of Research:

1. Optimization

1.1. Cell concentration

The optimized cell concentration of cell lysates was determined in the range of 10^5 to 10^6 cells/mL (**Figure 1**). Cell lysates concentrations lower than 10^5 to 10^6 cells/mL showed lower absorbance values (OD450 values) than OD450 values of blank controls.

Lysate concentration (cells/ml)	OD450			
	Unactivated THP-1		Activated THP-1	
	Batch 1	Batch 2	Batch 1	Batch 2
10^6	0.791 ± 0.099	0.640 ± 0.094	0.588 ± 0.032	0.580 ± 0.036
10^5	0.042 ± 0.009	0.009 ± 0.012	0.064 ± 0.012	0.052 ± 0.015
2×10^4	-0.011 ± 0.007	-0.025 ± 0.008	-0.002 ± 0.002	-0.009 ± 0.006
10^4	-0.015 ± 0.005	-0.015 ± 0.007	-0.009 ± 0.007	0.003 ± 0.018

Figure 1. *Evaluation of selected cell concentrations of THP-1 cell lysates with ELISA. Values highlighted in green depict an acceptable concentration range for further assays. Values depicted as mean ± SD. Evaluation of biological (n=2) and technical (n=3) replicates for each concentration level.*

1.2. LyP-1 concentration

The highest OD450 values of selected LyP-1 concentrations were measured at three LyP-1 concentrations - 500, 100 and 75µg/mL (**Figure 2**). Although the OD450 values of 100µg/mL LyP-1 concentration was not the highest, this LyP-1 concentration, demonstrated the lowest value distribution. Thus it was selected as the working LyP-1 concentration for the subsequent experimental procedures to provide consistency in measurements.

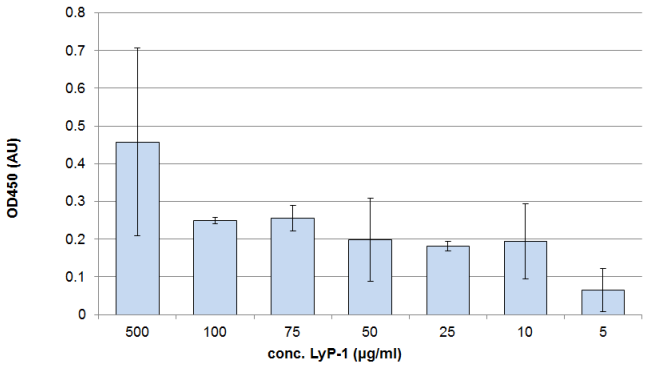

Figure 2.Evaluation of selected LyP-1 aptamer concentrations in ELISA. *10 ng/ml of human-source p32 standard was used per well. Values depicted as mean ± SD Evaluation of biological (n=2) and technical (n=3) replicates for each concentration level.*

1.3. LyP-1 binding affinity

The Lyp-1 aptamer was observed to have a lower OD450 value compared to the CT values at all tested concentrations of p32 standard protein (**Figure 3**). Nonetheless, the data from both, CT and LyP-1, also demonstrated a good fit ($R^2 > 0.99$). Moreover, the coefficient of variance (CV%) was observed to be low (CV < 10%).

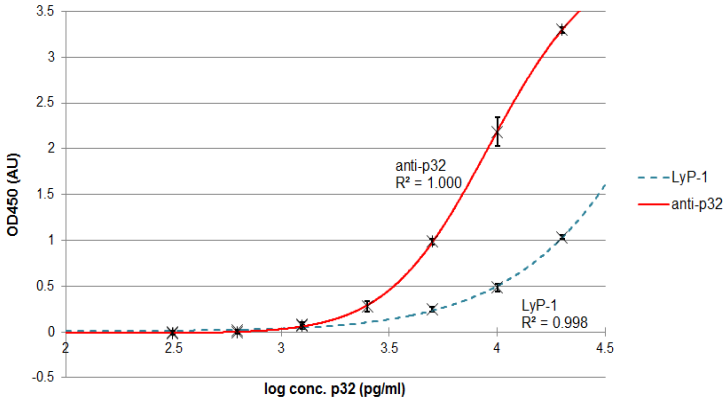

Figure 3. Evaluation of LyP-1 aptamer binding affinity. *Calibration curves produced by ELISA using either the anti-p32 antibody or LyP-1 aptamer. Values depicted as mean ± SD. Evaluation of biological (n=2) and technical (n=3) replicates for each point. Curves modelled with 4-parameter logistic regression (100 iterations), conducted with XLSTAT software.*

2. p32 interaction with CT and LyP-1 aptamer

2.1. Performance of LyP-1 aptamer against CT for cell-derived p32 protein

Overall, we observed a significantly higher absorbance values of LyP-1 aptamer in comparison to CT in all screened cell groups (non-activated and activated RAW264.7 and THP-1 cells) (**Figure 4**). Additionally, the RAW cells absorbance values were observed higher in comparison to THP-1 cells absorbance values. We speculated that the difference could be due to the CT being optimized for human-derived p32 protein, and not animal-derived p32 protein from cells such as the RAW264.7 cells and thus resulting in the higher signal difference observed in RAW264.7 cells.

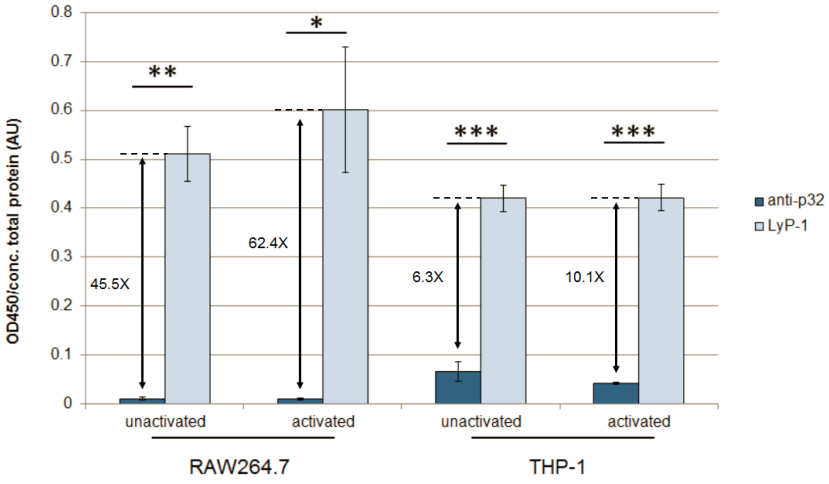

Figure 4. Evaluation of absorbance values of LyP-1 and CT in all cell groups. *Arrows represent the mean degree of signal increase. Values depicted as mean ± SD. Evaluation of biological (n=3) and technical (n=3) replicates for each cell group. Paired Student's t-test evaluated, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$*

As mentioned previously, our results indicated that the OD450 signal from the LyP-1 aptamer was significantly stronger than that of the CT in both non-activated and activated cells of both cell types, in contrast to the performance evaluation using p32 human standards. This contradiction could be possibly due to methodology differences such as cell lysis prior to the ELISA test. It is known that the trimeric structure of the p32 protein plays a key role in the binding of LyP-1. The cell lysis method applied in this study may have retained the trimeric structure, whereas this trimeric structure may not be present in the human protein standards used. Furthermore, replicating the process and purification method of the human standard is not possible due to proprietary information of the CT fabrication methods.

Future Areas for work:

The performance of the LyP-1 aptamer was demonstrated to be consistent in all executed experiments However, retrospectively, a temporal degradation in the LyP-1 aptamer was observed. We chose to keep to the same manufacturer to ensure consistency

of the LyP-1 aptamer source. The lengthy manufacturing and shipping time (4 – 6 weeks) of the LyP-1 aptamer was also a factor for us to keep to the same manufacturer. We would propose to explore new suppliers, or new methods of aptamer synthesis to obtain a LyP-1 aptamer with a longer shelf life.

We also propose more accurate methods such as flow cytometry. Researchers have utilised flow cytometry to determine the specificity of LyP-1 to target proteins [1] as well as to determine cell uptake of LyP-1 peptides [2]. Flow cytometry has also been used to detect cell-surface p32 expression [3], which is a closer representation of *in vivo* detection of atherosclerotic plaque. Imaging flow cytometry also allows for assessment of cell morphology on a single-cell basis [4], which will enable quantification of cell activation. We foresee that flow cytometry can provide better optimization results as well as experimental results.

Means of official announcement of Research Results:

Once the abovementioned future work has been accomplished, our plan is for submission of a manuscript for publication in a peer-reviewed journal such as Atherosclerosis by the publisher, Elsevier. Funding acknowledgment from MSIWF will be mentioned and a copy of the published manuscript will be given to MSWIF. The estimated time line for proper completion of the project and publication will be approximately 2 years.

References:

[1] Z. Wang *et al.*, "LyP-1 Modification To Enhance Delivery of Artemisinin or Fluorescent Probe Loaded Polymeric Micelles to Highly Metastatic Tumor and Its Lymphatics," *Molecular Pharmaceutics,* vol. 9, no. 9, pp. 2646-2657, 2012/09/04 2012.

[2] G. von Maltzahn *et al.*, "In Vivo Tumor Cell Targeting with “Click” Nanoparticles," *Bioconjugate chemistry,* vol. 19, no. 8, pp. 1570-1578, 07/09 2008.

[3] V. Fogal, L. Zhang, S. Krajewski, and E. Ruoslahti, "Mitochondrial/cell surface protein p32/gC1qR as a molecular target in tumor cells and tumor stroma," *Cancer research,* vol. 68, no. 17, pp. 7210-7218, 2008.

[4] L. F. Grimwade, K. A. Fuller, and W. N. Erber, "Applications of imaging flow cytometry in the diagnostic assessment of acute leukaemia," *Methods,* vol. 112, pp. 39-45, 2017/01/01/ 2017.