DESIGN, SYNTHESIS AND IN VITRO EVALUATION OF MODULATORS OF PFKFB3 AUTOREGULATORY DOMAIN

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INTRODUCTION

Atherosclerosis is a serious cardiovascular disease that can lead to severe complications such as myocardial infarction or stroke due to the rupture of an atherosclerotic plaque. Currently, two approaches are used in the therapy of atherosclerosis: i) manipulation of plasma lipoprotein or cellular cholesterol metabolism, ii) manipulation of inflammatory processes1. Although beneficial, recent therapeutic strategies have limited efficacy.2

It has been clearly shown that:
- pathological blood vessel responses are associated with metabolic alterations in endothelial cells (ECs)
- targeting EC glucose metabolism is a promising way to affect pathological angiogenesis3,4
- 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3)
  - plays a crucial role in the regulation of the EC glycolytic flux and it is up-regulated during angiogenesis5
  - is a dimeric bifunctional enzyme that possesses a very high kinase to phosphatase activity ratio
  - its activity is controlled by the N-terminus autoregulatory domain (AD) in the kinase region
- its crystal structure AD adopts a β-hairpin structure (Figure 1)6

We hypothesize that by interfering the interactions between the AD and bisphosphatase halt, we can indirectly achieve diminished kinase activity. The final biological outcome of this modulation would be reduced glycolysis.

DESIGN AND SYNTHESIS

a) DESIGN
Two strategies are suggested:
1. targeting the AD binding site for direct blockade of the interaction (Figure 2A)
2. targeting a negatively charged channel for an indirect interferance of the interaction (Figure 2B)

b) SYNTHESIS

All peptides selected from virtual screening (Table 1, HM 15-28) were synthesized using manual solid phase peptide synthesis or automated microwave assisted solid phase synthesis on several types of resins using Fmoc protected amino acids and standard protocols. Commercially available compounds (Table 1, HM 29-32) were purchased.

RESULTS

a) BINDING AFFINITY
The binding affinity of the selected compounds (Table 1) was determined using the microscale thermophoresis (MST). All compounds were tested in the concentration range from 1 mM to 30.25 nM (serial dilutions, 16 conc.). The measurements were repeated at least two times under the same conditions using 50 nM labeled enzyme.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Strategy</th>
<th>Binding Affinity (µM)</th>
</tr>
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<tbody>
<tr>
<td>HM-15</td>
<td>1/2</td>
<td>No binding</td>
</tr>
<tr>
<td>HM-16</td>
<td>1</td>
<td>No binding</td>
</tr>
<tr>
<td>HM-18</td>
<td>1</td>
<td>No binding</td>
</tr>
<tr>
<td>HM-19</td>
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<td>No binding</td>
</tr>
<tr>
<td>HM-20</td>
<td>1/2</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>HM-21</td>
<td>1/2</td>
<td>44 ± 14</td>
</tr>
<tr>
<td>HM-22</td>
<td>1/2</td>
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<tr>
<td>HM-27</td>
<td>2</td>
<td>No binding</td>
</tr>
<tr>
<td>HM-28</td>
<td>2</td>
<td>No binding</td>
</tr>
<tr>
<td>HM-29</td>
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</tr>
<tr>
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<td>1/2</td>
<td>No binding</td>
</tr>
<tr>
<td>HM-32</td>
<td>1</td>
<td>No binding</td>
</tr>
</tbody>
</table>

Table 1: Selected compounds.

Figure 1: Crystal structure of the human inducible form of PFKFB3 (the autoregulatory domain is shown in green).

Figure 2: Electrostatic surface representations of the virtual screening sites chosen for the two strategies. Negatively-charged patches are shown as red and positively-charged are in blue. The AD is shown in bright green. A: Surface shows the binding site of the AD (1st strategy). B: Surface shows the alternative targeting site (2nd strategy).

b) THERMOFLUOR
The thermal shift assay was carried out for PFKFB3 in the presence of the best binders (HM 20-22) and Sypro ORANGE dye was added. The experiment was done in 4 replicates in the temperature range from 4°C to 99°C.

Figure 3: Thermofluor analysis of the PFKFB3 in the presence of the compounds HM 20-22.

Figure 4: Cell migration assay for HM 20-22 at 18h.

D) CELL MIGRATION ASSAY
The scratch assay on murine ECs was performed for the compound HM 20-22. Each compound was tested at 10 µM and 100 µM concentrations. The result was checked after 18 (Figure 4) and 24 h (Figure 5).

Figure 5: Cell migration assay for HM 20-22 at 24h.

DISCUSSION

- The MST binding assay clearly shows that three compounds (HM 20-22) bind to the PFKFB3 enzyme in a low micromolar range.
- According to the MST results, a pharmacophore can be roughly determined, however further compounds have to be evaluated and the selected hits have to be optimized.
- Thermofluor analysis shows no significant change in the Tm of the target enzyme.
- The binding did not significantly affect the stability of the protein.
- Compound HM-21 was able to reduce the migration ability of murine ECs when compared to the control situation.

REFERENCES