Optimization of a Pulmonary Formulation of Tissue Plasminogen Activator for Pulmonary Delivery

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Objectives

• Discuss the rationale for creating a pulmonary formulation of tPA (pf-tPA).
• Present data supporting the feasibility of nebulizing pf-tPA.
• Discuss the effect of nebulization on the self-association of pf-tPA.
Tissue Plasminogen Activator

- Endogenous serine protease
- This protease is a significant contributor to the fibrinolytic pathway
  - Cleaves plasminogen to the fibrinolytically active form plasmin
- Primary clinical uses:
  - Dissolves thrombus associated with myocardial infarction and stroke
  - Also used to maintain catheter patency

Anti-Inflammatory Activity

- tPA also possesses anti-inflammatory activity that is:
  - Independent of fibrinolytic activity
  - Inhibits activator-induced oxidant production by neutrophils and macrophages
- tPA has been shown to inhibit inflammation in *in vivo* animal models
- This property of tPA could prove useful in the treatment of the Acute Respiratory Distress Syndrome (ARDS)
• ARDS is caused by damage to the alveolar epithelium and is characterized by:
  – Profound neutrophil infiltration into the lungs
  – Extensive inflammation and increased levels of proinflammatory cytokines (IL-1, TNFα)
  – Fibrin deposition which results in loss of lung compliance
• Currently there is no effective pharmacotherapy

(Left) RadiologyInfo Website. http://www.radiologyinfo.org/content/chest_radiography.htm
(Right) Evans, L. Norfolk and Norwich Hospital, Norwich, U.K. http://www.surgical-tutor.org.uk/default-home.htm?core/ITU/ards.htm~right

Need for a tPA Pulmonary Formulation
• IV administration of tPA for ARDS is not practical
  – IV administration results in disruption in coagulation homeostasis
• Pulmonary delivery would permit targeted delivery of tPA to the site of action
• Therefore, an optimal formulation was identified for pulmonary delivery
  – Protein must remain stable and active
  – Formulation must be safe and well tolerated
Protein Stability & Recovery

- Formulations were generated from Genentech’s Activase®
- Surfactant (Tween-80) was added to protein formulations in varying concentrations
- Stability and recovery was determined by UV absorbance at 280-400 nm
- tPA concentration range: 0.25-1 mg/mL
- Tween-80 surfactant concentration range: 0-0.5% (w/v)
- Prospective feasibility criteria were utilized to identify formulations feasible for nebulization.

Protein Stability & Recovery

- 27 formulations were screened for protein stability and recovery parameters
  - UV spectra were obtained before and after nebulization
  - Permitted determination of protein loss or structural change caused by nebulization
- Feasible formulations exhibited high protein recovery and an aggregation index (AI) < 10
- 15 formulations met feasibility criteria and exhibited protein recovery ≥75%
Biologic Activity

- Anti-inflammatory activity of nebulized vs. non-nebulized tPA was assessed
  - Neutrophils were isolated from human peripheral blood for the assay
  - Cells were exposed to tPA formulation for 60 min
  - Neutrophil superoxide anion ($O_2^{•−}$) production was induced by treatment of cells with phorbol myristate acetate (PMA)
  - $O_2^{•−}$ production of the neutrophils was determined by the rate of cytochrome c reduction
  - Feasibility criteria required at least a 50% reduction in $O_2^{•−}$ production

Anti-Inflammatory Activity

Formulation #1
1 mg/mL tPA; 0.05% Tween-80

• Suppression (84%) of PMA-induced $O_2^{•−}$ production
  - 1 mg/mL tPA; 0.05% Tween-80

Particle Size Determination

• Particle size of the aerosolized tPA formulations was determined by cascade impactor

  – The cascade impactor captures ranges of particle sizes on plates located in each stage of the instrument
  – Cascade impactor data were represented on log-probability plots and linear regression was performed for further analysis

Particle Size Determination

• Parameters determined from cascade impactor data:
  – Mass Mean Diameter (MMD)
  – Geometric Standard Deviation ($\sigma_g$)

• Ideal parameters of an aerosol respirable to the lower airways
  – MMD ≤ 5 µm
  – $\sigma_g$ > 1.2 µm

• Formulations exhibiting aerodynamic characteristics similar to these values were considered feasible
Particle Size Data

Formulation #1
1 mg/mL tPA; 0.05% Tween-80

Cumulative % Less Than Size Range

<table>
<thead>
<tr>
<th>Size Range</th>
<th>Cumulative %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 µm</td>
<td>99.9%</td>
</tr>
<tr>
<td>1 µm</td>
<td>99.99%</td>
</tr>
<tr>
<td>10 µm</td>
<td>99.99%</td>
</tr>
<tr>
<td>30 µm</td>
<td>99.99%</td>
</tr>
<tr>
<td>50 µm</td>
<td>99.99%</td>
</tr>
<tr>
<td>70 µm</td>
<td>99.99%</td>
</tr>
<tr>
<td>90 µm</td>
<td>99.99%</td>
</tr>
<tr>
<td>99 µm</td>
<td>99.99%</td>
</tr>
<tr>
<td>99.9 µm</td>
<td>99.99%</td>
</tr>
<tr>
<td>99.99 µm</td>
<td>100%</td>
</tr>
</tbody>
</table>

• Formulation 1:
  – MMD = 2.4 µm ($\sigma_g$ = 2.7 µm)


Conclusions

• 15 formulations remained stable following nebulization and exhibited >75% protein recovery
• pf-tPA formulation #1 reduced PMA-induced superoxide anion production from human neutrophils by >50%
• Formulation #1 exhibited optimal particle size distribution for aerosol pulmonary delivery
Optimization of Aerosol Collection

- Collection of pf-tPA induced ~25% insoluble aggregation
  - Efforts were made to optimize this collection method
- Nebulization-induced protein self-associations were quantitated by analytical ultracentrifugation (AUC)
- Biologic mechanism of pf-tPA anti-inflammatory activity is unknown and is being studied

pf-tPA Aerosol Collection

- Collection of pf-tPA induced ~25% insoluble aggregation
- Modified collection system permitted nebulization of protein, cooling in a condenser coil, and collection on silicon tubing
  - Insoluble aggregation was eliminated
- This method could be an effective means of collecting and analyzing aerosolized protein formulations
pf-tPA Aerosol Collection

<table>
<thead>
<tr>
<th>Sample</th>
<th>Formulation</th>
<th>Post Nebulized Recovery</th>
<th>Aggregation Index (AI)</th>
<th>Percent Insoluble Aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flask Condenser System</td>
<td>1 mg/mL tPA; 0.05% Tween-80</td>
<td>0.71 mg/mL</td>
<td>2.8</td>
<td>23.5%</td>
</tr>
<tr>
<td>Condenser Coil System</td>
<td>1 mg/mL tPA; 0.05% Tween-80</td>
<td>1.27 mg/mL</td>
<td>4.4</td>
<td>None detectable</td>
</tr>
</tbody>
</table>

Self-Association Assessment

- Second derivative UV spectroscopy does not identify the nature of soluble aggregation
- Sought to determine the nature of soluble aggregates as a function of nebulization
  - Used AUC to accomplish this
  - Advantageous since the formulation can be assessed at 1 mg/mL in formulation buffer
Sedimentation Velocity of pf-tPA

- Prenebulized and nebulized pf-tPA was assessed by AUC
  - Three concentrations assessed (4, 8, and 16 µM)
- Sedimentation velocity was performed to assess self-association as a result of nebulization
  - Performed in a Beckman Coulter XL-A
  - Protein sedimented at 50,000 rpm, 4°C, 295nm
- Data fit performed for c(s) distribution with Sedfit 9.4 (Peter Schuck, NIH, http://www.analyticalultracentrifugation.com)

Analytical Ultracentrifuge Schematic

Image Reference:
Ralston, G. Introduction to Analytical Ultracentrifugation. Univ. of Sydney. Sydney, Australia
Sedimentation Velocity Results

![Graph showing sedimentation coefficients for different conditions.]

- Prenebulized pIFPA (4 uM)
- Nebulized pIFPA (4 uM)
- Prenebulized pIFPA (8 uM)
- Nebulized pIFPA (8 uM)
Sedimentation Velocity Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sedimentation Coefficient (s)</th>
<th>Frictional Ratio ($f/f_0$)</th>
<th>RMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 µM pf-tPA (Prenebulized)</td>
<td>2.34</td>
<td>1.41</td>
<td>4.87 x 10^{-3}</td>
</tr>
<tr>
<td>4 µM pf-tPA (Nebulized)</td>
<td>2.30</td>
<td>1.44</td>
<td>6.32 x 10^{-3}</td>
</tr>
<tr>
<td>8 µM pf-tPA (Prenebulized)</td>
<td>2.36</td>
<td>1.47</td>
<td>6.73 x 10^{-3}</td>
</tr>
<tr>
<td>8 µM pf-tPA (Nebulized)</td>
<td>2.30</td>
<td>1.34</td>
<td>7.43 x 10^{-3}</td>
</tr>
<tr>
<td>16 µM pf-tPA (Prenebulized)</td>
<td>2.33</td>
<td>1.41</td>
<td>8.59 x 10^{-3}</td>
</tr>
<tr>
<td>16 µM pf-tPA (Nebulized)</td>
<td>2.25</td>
<td>1.36</td>
<td>9.04 x 10^{-3}</td>
</tr>
</tbody>
</table>
Conclusions

- pf-tPA stability and recovery was improved by using a coil-condenser collection system
- pf-tPA remains monomeric following nebulization as determined by AUC
  - Nebulization of this protein formulation does not induce self-association
- Immunogenicity of pf-tPA will be determined by an in vivo study

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