Development of a Real Time PCR-based Bioassay for Human Interferon β

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Presentation Outline

• Interferon $\beta$ (IFN$\beta$) – Pharmacology
• IFN$\beta$ Specific Activity - Measurement
• RT-PCR Assay - Development Approach
• Results
• Data comparison
• Conclusions
Pharmacology of IFNβ

- Interferons are a family of naturally occurring proteins and glycoproteins that are produced by eukaryotic cells in response to viral infection and other biological inducers.
- IFNβ is produced by various cell types including fibroblasts and macrophages.
- Interferons mediate antiviral, antiproliferative and immunomodulatory activities in response to viral infection and other biological inducers.
Pharmacology of IFNβ

• IFNβ exerts its biological effects by binding to specific receptors on the surface of human cells (IFNAR).

• The specific interferon-induced proteins and mechanisms by which IFNβ exerts its effects in multiple sclerosis have not been fully defined.

• The binding of IFNβ initiates a complex cascade of intracellular events that leads to the expression of numerous interferon-induced gene products and markers, including 2', 5'-oligoadenylate synthetase (OAS), β2-microglobulin, Myxovirus resistance proteins (MxA) and neopterin.

• These products have been measured in the serum and cellular fractions of blood collected from patients treated with IFNβ-1a (AVONEX®).
Pharmacology of IFNβ

IFNβ Proposed Mechanism of Action

IFN-induced proteins
- Antiviral response
- β2-microglobulin
- GTP-cyclohydrolase (neopterin)
- Mx proteins
- Many others (efficacy in MS)

Cell membrane

Cellular effects of IFNβ administration.

Pachner, AR, Multiple Sclerosis 2007; 13: S49-S52
IFNβ-1a Specific Activity

• **Standard** - World Health Organization (WHO) natural interferon beta standard, Second International Standard for Interferon, Human Fibroblast (Gb-23-902-531),

• **Assay** - *in vitro* CytoPathic Effect bioassay (CPE) using lung carcinoma cells (A549) and EncephaloMyoCarditis virus (EMC).

• IFNβ–1a (AVONEX®) contains approximately 200 million IU of antiviral activity per mg, using this method.
Clinical application of the CPE Assay

- Bioavailability of IFNβ in human serum (PK, NAbs)
- Measured by ELISA or CPE
- Sensitivity - CPE assay vs. ELISA
- Levels of IFNβ–1a after dose are often too low to be measured in standard ELISA
CPE Assay

Based on the ability of IFNβ to protect human lung carcinoma cells (A549) from cytopathic effect (CPE) caused by the Encephalomyocarditis (EMC) virus.

Day 1: A549 cells are added to the wells of an assay plate(s). Plates are incubated for 20-24 hours at 37±1 °C / 5%±1 % CO2.

Day 2: Standards, samples and controls are added to the assay plates and incubated for 15-20 hours.

Day 3: The EMC virus is added to all standard, samples, controls and virus control wells and incubated further for 30±1 hours.

Day 4: Colorimetric substrate is added to all the assay plates and incubated at 37±1 °C / 5% CO2 for additional 2 to 4 hours. Plate reader is used to record absorbance at 490 nm.

‘Pros’ and ‘Cons’ of CPE Assay

**‘Pros’**
- ‘Low tech’ assay – no specialized equipment is needed (but specialized personnel and lab is)
- Relatively low cost of reagents

**‘Cons’**
- Time consuming
- Poor reproducibility
- Many variables
- Narrow range of Standard Curve
- Poor sensitivity
- Difficult outsourcing
IFNβ-1a Standard Curve in 10% Human Serum

(MTS-colorimetric read-out)
Assay range: ~1-5 IU/mL
MxA gene expression in response to IFNβ

Several assays based on this mechanism are described in literature

• **MxA protein induction assay (MPA)** - quantifying an antiviral protein (MxA for myxovirus resistant protein A) specifically induced by IFNβ
  


• **Neutralizing antibody detection in real time based PCR assay**

IFNβ: qRT-PCR Assay Design

Based on the ability of IFNβ to bind to the specific receptors on human lung carcinoma cells (A549) and induce cascade of intracellular events resulting in up-regulation of specific genes (MxA, OAS, etc).

Day 1: A549 cells are added to the wells of an assay plate(s). Plates are incubated overnight at 37±1 °C / 5±1% CO2.

Day 2: IFNβ is added to the assay plates and incubated for 4-5 hours. Cells are lysed and TaqMan reactions are performed directly on the cell lysates or, alternatively extracted RNA is Reverse-Transcribed overnight and,

Day 3: TaqMan reactions are performed on cDNA.
‘Pros’ and ‘Cons’ of qRT-PCR Assay

‘Pros’

• Capturing ‘early event’ (mRNA levels) = reduced variability
• Normalizing with internal Endogenous control (GAPDH) = control for well to well variability due to different cell densities, RNA isolation and reverse transcription efficiencies and RNA degradation
• Pre-optimized reagents readily available
• Reduced processing time and increased throughput
• Increased Standard curve range and sensitivity

‘Cons’

• Specialized equipment is needed to perform the assay
• Cost of initial setup (equipment and training)
• Cost of reagents
Principle of qRT-PCR

Two-step RT-PCR

- **Extension of primer on mRNA**
  - 5' mRNA
  - 3' cDNA

- **Synthesis of 1st cDNA strand**
  - 3' cDNA

- **PCR Step**
  - **Extension of primer on cDNA**
    - 3' Forward Primer
    - 5' Reverse Primer
  - **Completion of 2nd cDNA strand**
    - 5' Forward Primer
    - 3' Reverse Primer
  - **PCR amplification of cDNA**
    - 3' cDNA
    - 5' cDNA

- **Cycle #1**
- **Cycle #2**

The 5’-Nuclease Assay - TaqMan

- **Polymerization**
  - Forward Primer
  - TaqMan MGB probe
  - Reverse Primer

- **Strand Displacement**

- **Cleavage**

- **Completion of Polymerization**

- **Nonfluorescent quencher**
- **MGB** = Minor groove binder
- **R** = Reporter
- **E** = AmpliTaq Gold DNA Polymerase, UP
Analysis of Real-Time PCR Data

Experimental Data

ABI Prism 7900HT
Sequence Detection System (SDS)

Low CT = more RNA
High CT = less RNA
1 CT = 2-fold difference in amount of template

C_t = 29.1
Absolute vs. Relative Quantitation

- **Absolute quantitation** requires that the absolute quantities of the standard be known by some independent means.

- Plasmid DNA or *in vitro* transcribed RNA are commonly used to prepare absolute standards. Concentration is measured by A260 and converted to the number of copies using the molecular weight of the DNA or RNA.

- **Relative quantitation** - quantity is expressed relative to some basis sample, such as the calibrator. For all experimental samples, target quantity is determined from the standard curve and divided by the target quantity of the calibrator. Thus, the calibrator becomes the 1 × sample, and all other quantities are expressed as an n-fold difference relative to the calibrator. For example, in a study of drug effects on gene expression, the untreated control would be an appropriate calibrator.

- **ΔΔCₜ method** - Relative quantitation can be performed using this method instead of relative standard curve. It is necessary to demonstrate that efficiencies of target and reference amplification are approximately equal.
Principle of Relative Quantitation

- Amplification of an endogenous control is performed to standardize the amount of sample RNA or DNA added to a reaction. Examples of such genes are β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA (18S).

- **Endogenous control** – an RNA or DNA that is present in each experimental sample as isolated. By using an endogenous control as an active reference, you can normalize quantitation of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.

- **Calibrator** - A sample used as the basis for comparative results.
Normalization with Endogenous Control (GAPDH)

Bi-plex reactions: Determination of MxA levels normalized with GAPDH

MxA response
GAPDH levels

Experimental Data
CPE qRT-PCR Assay Development

- Targets:
  - IFNβ−1a
  - Modified IFNβ−1a

- Matrix effect
  - Normal Human Serum (NHS)

- Time-course

- OAS2 vs. MxA expression

- Two step vs. One step read-out

- Assay performance - Clinical sample testing
Matrix Effect: IFNβ-1a

NHS effect (2-10%) on IFNβ-induced MxA expression

Better dynamic range (10%)

Superimposed curves (2% and 5%)

Matrix Interference

4-P Fit: \( y = (A - D)\frac{1}{(1 + (x/C)^B)} + D \)

<table>
<thead>
<tr>
<th>Plot#</th>
<th>(IFN-beta 1a 2%: Concentration vs Values)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>R²</th>
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<td>0.999</td>
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<td>44.6</td>
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</table>
Matrix Effect: Modified IFNβ-1a

NHS effect (2-10%) on IFNβ-induced MxA expression

Better dynamic range (10%)

Comparable curves (2% and 5%)

4-P Fit: $y = \frac{A - D}{(1 + (x/C)^B)^D} + D$

<table>
<thead>
<tr>
<th>Plot#1 (Modified IFN-beta 1a 2%: Concentration vs ...</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>R^2</th>
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<th>B</th>
<th>C</th>
<th>D</th>
<th>R^2</th>
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<th>Plot#3 (Modified IFN-beta 1a 10%: Concentration v...</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>R^2</th>
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<td>25.1</td>
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Time-course of IFNβ-1a induced MxA expression

MxA expression after 2, 3, 5 and 24 hrs of exposure

5 hrs – best sensitivity and dynamic range

4-P Fit: $y = \frac{(A - D)}{1 + \left(\frac{x}{C}\right)^B} + D$

<table>
<thead>
<tr>
<th>Plot#1 (IFN-beta 1a 2 hrs: Concentration vs Values)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>R^2</th>
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<tr>
<td>-0.244</td>
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<td>Plot#3 (IFN-beta 1a 5 hrs: Concentration vs Values)</td>
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<td>267</td>
<td>97.9</td>
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Time-course of Modified IFNβ–1a induced MxA expression

MxA expression after 2, 3, 5 and 24 hrs of exposure

5 hrs – best sensitivity and dynamic range
IFNβ-1a induced MxA and OAS2 expression

OAS2 expression

MxA expression

### IFN-beta 1a 10% Serum, 5 hrs - OAS2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Values</th>
<th>%Return</th>
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</thead>
<tbody>
<tr>
<td>S1</td>
<td>80,000</td>
<td>12,693</td>
<td>114.618</td>
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<tr>
<td>S2</td>
<td>80,000</td>
<td>12,028</td>
<td>110.326</td>
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<tr>
<td>S3</td>
<td>40,000</td>
<td>5,902</td>
<td>33.033</td>
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<tr>
<td>S4</td>
<td>20,000</td>
<td>5,416</td>
<td>25.847</td>
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<tr>
<td>S5</td>
<td>10,000</td>
<td>3,608</td>
<td>16.316</td>
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<tr>
<td>S6</td>
<td>5,000</td>
<td>1,960</td>
<td>9.100</td>
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<tr>
<td>S7</td>
<td>2,500</td>
<td>0.931</td>
<td>4.562</td>
</tr>
<tr>
<td>S8</td>
<td>1,250</td>
<td>0.398</td>
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<td>S9</td>
<td>0.625</td>
<td>0.064</td>
<td>0.305</td>
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<tr>
<td>S10</td>
<td>0.313</td>
<td>0.027</td>
<td>0.106</td>
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<td>S11</td>
<td>0.158</td>
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<tr>
<td>S12</td>
<td>0.079</td>
<td>0.016</td>
<td>0.272</td>
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### IFN-beta 1a 10% Serum, 5 hrs - MxA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Values</th>
<th>%Return</th>
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</thead>
<tbody>
<tr>
<td>S1</td>
<td>100,000</td>
<td>138.747</td>
<td>39.820</td>
</tr>
<tr>
<td>S2</td>
<td>80,000</td>
<td>111.137</td>
<td>35.612</td>
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<tr>
<td>S3</td>
<td>40,000</td>
<td>40.015</td>
<td>29.254</td>
</tr>
<tr>
<td>S4</td>
<td>20,000</td>
<td>19.841</td>
<td>21.081</td>
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<tr>
<td>S5</td>
<td>10,000</td>
<td>10.882</td>
<td>14.773</td>
</tr>
<tr>
<td>S6</td>
<td>5,000</td>
<td>5.354</td>
<td>7.042</td>
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<tr>
<td>S7</td>
<td>2,500</td>
<td>2.402</td>
<td>4.305</td>
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<tr>
<td>S8</td>
<td>1,250</td>
<td>1.260</td>
<td>1.676</td>
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<tr>
<td>S9</td>
<td>0.625</td>
<td>0.490</td>
<td>0.601</td>
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<tr>
<td>S10</td>
<td>0.313</td>
<td>0.300</td>
<td>0.215</td>
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<td>S11</td>
<td>0.158</td>
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<tr>
<td>S12</td>
<td>0.079</td>
<td>0.233</td>
<td>0.051</td>
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Modified IFNβ–1a induced MxA and OAS2 expression

OAS2 expression

MxA expression
One Step vs. Two Step read-out: IFNβ-1a

‘One-Step’ process:
Cell Lysis > One Step RT-PCR

‘Two-Step’ process:
RNA isolation > RT > TaqMan

IFN-beta 1a Standards 1 (IU/ml)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Values</th>
<th>Backfit</th>
<th>%Return</th>
</tr>
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<tbody>
<tr>
<td>Av01</td>
<td>460,000</td>
<td>2136.17</td>
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<tr>
<td>Av02</td>
<td>64,000</td>
<td>Masked</td>
<td>Masked</td>
<td>Masked</td>
</tr>
<tr>
<td>Av03</td>
<td>25,600</td>
<td>1119.01</td>
<td>25.159</td>
<td>98.3</td>
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<tr>
<td>Av04</td>
<td>10,240</td>
<td>638.46</td>
<td>10.657</td>
<td>104.07</td>
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<tr>
<td>Av05</td>
<td>4,096</td>
<td>205.39</td>
<td>4.109</td>
<td>100.31</td>
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<tr>
<td>Av06</td>
<td>1,638</td>
<td>84.077</td>
<td>1.337</td>
<td>81.50</td>
</tr>
<tr>
<td>Av07</td>
<td>0.665</td>
<td>21.828</td>
<td>0.665</td>
<td>92.37</td>
</tr>
<tr>
<td>Av08</td>
<td>0.262</td>
<td>8.529</td>
<td>0.468</td>
<td>174.70</td>
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One Step vs. Two Step read-out: Modified IFNβ-1a

‘One-Step’ process:
Cell Lysis > One Step RT-PCR

‘Two-Step’ process:
RNA isolation > RT > TaqMan
Optimized Assay Design

- Optimum IFNb exposure time – 4-5 hrs
- Optimum NHS concentration – 10%
- Assay performs equally well for IFNβ-1a and modified IFNβ-1a (sensitivity, dynamic range)
- OAS2 read-out has similar sensitivity and range as MxA, but MxA is chosen (literature references)
- Read-out can be performed either as One-step (lysis - TaqMan) or Two-step (RNA - cDNA - TaqMan)
Clinical Samples

- 10 patients / 4 time points each
  - pre-, 6 hrs, 12 hrs, 24 hrs
  - historical data available (CPE assay)

Levels of IFN-beta 1a in Patient Serum Samples - Comparison of Two Methods

- Comparable PK profiles
- Improved Sensitivity
Conclusions

• The IFNβ PCR based assay is
  – sensitive
  – simple
  – time efficient
  – has significant advantages over the established CPE assay.

The assay is easily transferable to a commercial testing laboratory and could be used for product release and in a clinical setting.
Acknowledgments

• Gulraiz Khan
• Michaela Lerner
• Paula Hochman
• Meena Subramanyam
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