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Biological response genes after single dose administration of interferon β-1b to healthy male volunteers

Jan Hilpert [a], Johanna M. Beekman [a], Susanne Schwenke [b], Kristin Kowal [a], David Bauer [a], Johannes Lampe [c,1], Rupert Sandbrink [c], Jürgen F. Heubach [a], Steffen Stürzebecher [a,2,3], Joachim Reischl [a,*,3]

[a] *Clinical Pharmacology, Bayer Schering Pharma AG, Berlin, Germany*
[b] *Global Drug Discovery Statistics, Bayer Schering Pharma AG, Berlin, Germany*
[c] *Global Clinical Development Specialized Therapeutics, Bayer Schering Pharma AG, Berlin, Germany*

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Abstract

Treatment with interferon β-1b (IFNB-1b) is clinically effective in multiple sclerosis patients. However, the mechanism of action is only partially understood, and validated biological response markers are lacking. We assessed IFNB-1b-induced transcriptional changes by microarray technology. Healthy male volunteers received 250 μg IFNB-1b or placebo in a double-blind, randomized controlled trial ($n=5$ per group). Most transcripts demonstrated peak levels after 6–12 h and returned to baseline after 48 h. We identified 227 differentially regulated genes including novel and previously described markers. This panel may become a valuable tool for development of new IFNB-1b formulations and assessment of clinical drug effects.

Keywords: Gene expression; Biomarker; Interferon beta; Time course; MxA; Metallothionein

1. Introduction

The clinical efficacy of IFNB-1b in reducing the relapse rate of multiple sclerosis (MS) patients has been well established for more than 10 years (IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1995). Recently, it has been shown that early treatment of patients with IFNB-1b after a first event suggestive of MS (clinically isolated syndrome, CIS) is beneficial with regard to delaying the time to clinically definite MS (Kappos et al., 2006). Furthermore, early treated CIS patients showed reduced disability at three years after initiation of treatment compared to CIS patients with delayed IFNB-1b treatment (Kappos et al., 2007). Despite these clinical benefits, the precise mechanism of action of IFNB-1b is still unknown. Validated biological response markers that could be used for dose-finding studies or bioequivalence studies are lacking. We employed the microarray technology to address this need by gene expression profiling in healthy male volunteers that either received a single dose of IFNB-1b or placebo, respectively.

The microarray technology has been used previously in various studies to investigate the short term effects of interferon-β on the gene expression pattern in peripheral blood (Wandinger et al., 2001; Stürzebecher et al., 2003; Weinstock-Guttman et al., 2003; Satoh et al., 2006; Fernald et al., 2007; Singh et al., 2007; Rani et al., 2007). With one exception (Fernald et al., 2007), these groups focused on the analysis of peripheral blood mononuclear cells (PBMC), rather than on

* Corresponding author. Clinical Biomarkers, Clinical Pharmacology, Bayer Schering Pharma AG, Müllerstr. 178, D-13442 Berlin, Germany. Tel.: +49 30 468 11788; fax: +49 30 468 91788.
E-mail address: joachim.reischl@bayerhealthcare.com (J. Reischl).
[1] Current affiliation: Lampe & Konieczny GmbH & Co. KG, Berlin, Germany.
[2] Current affiliation: Grünenthal GmbH, Aachen, Germany.
[3] Equally contributing senior authors.

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whole blood. Many of these studies were based on *in vitro* incubation of blood cells with recombinant interferon-β rather than on *in vivo* exposure.

The concept and design of the present study are novel for several reasons. First, the gene expression profiling investigations were carried out on samples obtained from healthy male volunteers being administered a single dose of IFNB-1b in a highly standardized phase 1 setting. Secondly, we included a placebo group to control for placebo response and other potential confounders such as diurnal effects. Finally, we used a sampling system specifically designed to capture the RNA expression profile in whole blood (PAXgene; Rainen et al., 2002). This approach prevents the introduction of a bias due to different PBMC preparation techniques used at various sites and hence allows technical transfer into late phases of clinical development.

The aim of the current study was to identify an *in vivo* gene expression signature modulated by IFNB-1b in whole blood samples and to characterize the kinetics of the gene expression changes. We report the expression kinetics of biological response genes and define a panel of 267 transcripts regulated by IFNB-1b in treatment-naïve healthy male volunteers after single dose administration of IFNB-1b.

2. Materials and methods

2.1. Study design

This study was performed according to the principles outlined in the declaration of Helsinki after approval by the local Institutional Review Board. Five healthy male volunteers (age range 32–38 years) received 250 μg IFNB-1b (8 MIU Betaferon®, Betaseron®, subcutaneous injection) and five healthy male volunteers (age range 30–42 years) were administered placebo in a double-blind, randomized manner. Blood samples for expression profiling were taken using the PAXgene system (Qiagen, Hilden, Germany) before injection and at 3, 6, 12, 24, 48 and 96 h post injection. In addition, blood cell counts were carried out before injection and at 12, 24, 36, 48, 72 and 96 h post injection.

2.2. Microarray gene expression profiling

Total RNA was isolated from the PAXgene blood samples according to the manufacturer's protocol (Qiagen, Hilden, Germany). RNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, NC). RNA quality and purity were assessed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA).

To reduce an excess of hemoglobin RNA, the RNA samples were pretreated according to the Affymetrix globin depletion procedure using oligonucleotides for the human alpha1, alpha2 and beta hemoglobin genes (Affymetrix, Santa Clara, CA). Basically, the oligonucleotides were annealed to 3 μg of total RNA. The resulting RNA/DNA hybrids were digested with RNAse H and the remaining RNA was cleaned up using a GeneChip sample cleanup module (Affymetrix). This RNA was used in the labelling procedure using the One-Cycle Target Labeling and Control Reagents from Affymetrix. The hemoglobin depleted RNA was first reverse transcribed using a T7-$(dT)_{24}$ primer. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA was purified and served as a template in the subsequent *in vitro* transcription (IVT) reaction. The IVT reaction was carried out in the presence of T7 RNA polymerase and a biotinylated ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labelling. The biotinylated cRNA targets were then cleaned up. Subsequently, the labeled cRNA was quantified using the NanoDrop spectrophotometer and checked for quality on the Agilent Bioanalyzer. An aliquot of 20 μg labeled cRNA was fragmented, and 8 μg of the fragmented cRNA was then hybridized to a HG-U133A array (containing nearly 23,000 probe sets representing 14,500 well characterized transcripts and variants). The arrays were washed, stained and scanned (GeneChip-3000 Scanner, Affymetrix) resulting in a scanned image (∗.DAT files). These files were quantitated using the GCOS software by Affymetrix resulting in ∗.CEL files. The CEL files were condensed using the Affymetrix MAS 5.0 algorithm. Quality control of the GeneChip experiments was performed by assessing the background, noise, percent present call, GAPDH 3′/5′ ratio, the hybridization controls and the scaling factors of all GeneChips applying the limits recommended by the manufacturer.

2.3. RT-PCR gene expression profiling

For confirmation studies with real-time polymerase chain reaction (RT-PCR), total RNA from the same RNA population was used as for the microarray gene expression profiling experiments. First strand cDNA was synthesized from 500 ng total RNA with the Superscript First Strand Synthesis System for RT-PCR using an oligo $(dT)_{12-18}$ primer (Invitrogen Life Technologies, Carlsbad, USA) in a 20 μl reaction volume. One μl of these reactions were used to analyse the expression of the target genes. PCR was performed on a TaqMan 7700 (Applied Biosystems) using the following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, USA): GAPDH (Hs00266705_g1), GBP1 (Hs00977005_m1), IRF7 (Hs01014809_g1), GCH1 (Hs00609198_m1), OAS-like (Hs00984390_m1), STAT1 (Hs01014002_m1), MxA (Hs00182073_m1), MxB (Hs01550808_m1). The reactions were performed in 25 μl using a qPCR Mastermix (Eurogentec, Köln, Germany) under the following thermal cycling conditions: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 2 min. Measurements were performed in duplicates. All duplicates which had a larger than 0.5 C_T difference were excluded from analysis. The relative quantification of each target mRNA normalized with the GAPDH endogenous control was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). All assays showed amplification efficiencies of 90–110%.

2.4. Statistics

Analysis of expression data was performed by using the Expressionist Analyst Version 4.2.3 (GeneData AG, Basel,