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Quantitative and qualitative pleiotropic differences between Simvastatin single and Vytorin combination therapy in hypercholesterolemic subjects

Zohara Sternberg [a,*], Trevor Chichelli [a], Daniel Sternberg [a], David Hojnacki [a], Allison Drake [a], Song Liu [b], Qiang Hu [b], Frederick Munschauer [a]

[a] Department of Neurology, Stroke Center, Buffalo Medical Center, Buffalo, NY, USA
[b] Department of Bioinformatics, Roswell Park Cancer Institute, Carlton Street, Buffalo, NY, USA

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ABSTRACT

Aims: This cross-sectional study tested the hypothesis that treatment with the combination of Ezetimibe/Simvastatin (Vytorin) leads to broader changes in the expression levels of immunomodulatory genes as compared to Simvastatin monotherapy.
Methods: Illumina's GenomeStudio gene expression module was used to compare gene profiles of Vytorin and Simvastatin in the peripheral blood mononuclear cells of 20 hypercholesterolemic subjects.
Results: The characteristics of the immunomodulatory genes, which were altered by Vytorin, differed from those genes which were altered by Simvastatin. Vytorin mostly altered the expression levels of genes related to inflammation/oxidative stress; it downregulated the *NF-KappaB* and upregulated the expression of anti-inflammatory cytokine, *IL-10*, and anti-oxidant enzymes, *GPX1* and *SOD2*, but also upregulated the expression levels of genes involved in cellular activation, adhesion, and coagulation cascade, including *VWF*, *F7*, *PF4*, *PF4V1 SELP*, *ITGB3*, *ITGB5*.
Simvastatin mostly altered the expression levels of genes related to cellular apoptosis/proliferation. It upregulated the expression levels of apoptosis-related genes *APAF1*, *BAX*, *IER3*, and *CSF1R*, and downregulated the expression levels of genes related to cellular proliferation, including *PTN* and *CD69*. Treatment with Vytorin combination therapy modulated lipid profile and serum levels of the C-reactive protein more effectively, than treatment with Simvastatin monotherapy.
Conclusion: The nature of the pleiotropic effects may play a role in Vytorin's and Simvastatin's clinical efficacies.

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1. Introduction

Both hyperlipidemia/hypercholesterolemia [1] and inflammation/oxidative stress [2,3] contribute to atherosclerosis and to cerebrovascular events. Similar to Simvastatin (Sim), Ezetimibe (Zetia) is an effective hypolipidemic/hypocholesterolemic agent [4]. Furthermore, the administration of Vytorin (Vyt) (10 mg Zetia+20 mg Sim) to hypercholesterolemic patients leads to an additive effect in lowering LDL-C (55%) compared to monotherapy with Zetia (20%) or Sim (38%) [5].

Similar to statins [6], Zetia possesses pleiotropic effects beyond its lipid lowering effects, including anti-inflammatory and anti-oxidant effects [7–10]. Daily treatment of hypercholesterolemic patients with 40 mg Sim monotherapy or in combination with 10 mg Zetia, for up to 90 days, showed reduced monocyte release of inflammatory cytokines, and chemokines, and reduced C-reactive protein (CRP) serum levels compared to placebo. Combination therapy further reduced the release of cytokines from monocytes and CRP serum levels to levels observed in healthy controls [11]. Similar results were observed after a 12-week daily treatment of hypercholesterolemic patients with 10 mg Zetia/10–80 mg Sim combination therapy, showing further reduction in CRP serum levels at each individual Sim dose [12].

Abbreviations: ACE, angiotensin-converting enzyme; ACS, acute coronary syndrome; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ARB, angiotensin receptor blockers; BP, blood pressure; CHD, coronary heart disease; CIMT, carotid intima-media thickness; CRP, c-reactive protein; HDL, high-density lipoprotein cholesterol; IL, interleukin; LDL, low-density lipoprotein cholesterol; *NFk-B*, nuclear factor kappaB; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxygen species; *VWF*, von Willebrand factor; TC, total cholesterol; *TNF*, tumor necrosis factor; TRG, triglyceride.

* Corresponding author. Stroke Center, Buffalo Medical Center, Buffalo, NY 14203, USA. Tel.: +1 716 859 4235; fax: +1 716 859 2430/7573.
E-mail address: zs2@buffalo.edu (Z. Sternberg).

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Furthermore, a crossover study in hypercholesterolemic patients shows the efficacy of both Zetia/Sim (10 mg/10 mg) combination therapy and Sim (80 mg) monotherapy in reducing endothelial dysfunction, CRP serum levels, and LDL-C plasma levels [13].

Nevertheless, a six-week Zetia/Sim (10 mg/20 mg) combination therapy or Sim (80 mg) monotherapy of patients with coronary artery disease (CAD) who were initially on chronic 20 mg Sim regimen showed no incremental effects on circulating endothelial progenitor cells, a surrogate marker of endothelial function, despite further reduction in TC [14].

This crossover study compared the changes in gene expression profiles (with an emphasis on changes in immunomodulatory genes) in the peripheral blood mononuclear cells (PBMCs) of hypercholesterolemic subjects, after combination therapy with Vyt or monotherapy with Sim. In addition, the relationships between CRP serum levels, gene expression levels, and lipid profiles were examined.

2. Materials and methods

2.1. Population

Twenty (20) hypercholesterolemic subjects (11 males), otherwise healthy subjects, with no CAD history, mean age 46.4 ± 8.5 years, including, who had total cholesterol >200 mg/dL and/or LDL-C >130 mg/dL were recruited from the Primary Care, Kaleida Health Medical Center, Buffalo, NY. Patients' exclusion criteria were as followings: Patients treated with statins, Zetia or Vyt less than six months previous to the blood draw, patients with allergic reaction or any other contraindications to Zetia/Sim, patients with active liver disease indicated by prescreening elevated levels of liver enzymes, aspartate transaminase (AST) or alanine transaminase (ALT) > 45 IU/L, patients taking immunosuppressive drugs, diabetic patients, patients taking drugs that inhibit CYP3A4, or patients on calcium channel blockers and patients with chronic renal disease with glomerular filtration rate (GFR) < 35 mL/min.

2.2. Design

In this 18-week randomized crossover study, all 20 patients were treated with either a combination of 40 mg Sim and 10 mg Zetia (Vyt) or 40 mg Sim single for six weeks. After the initial six-week treatment with the first drug, patients were subjected to a six-week wash out period, and subsequently were treated with the second drug. The order of drug administration was randomized and was based on odd and even numbers. The wash out period was designed to eliminate treatment effects and return patients to baseline gene expression levels. Blood samples were obtained at the two baselines, and post each drug treatment for gene expression analysis, determination of lipid profiles, and for the measurements of CRP serum levels, and liver aminotransferases.

2.3. Logic behind the selection of mononuclear cells

Gene expression was studied in the PBMCs, since these cells resemble hepatocytes in regard to the expression of genes involved in cholesterol metabolism [15]. In addition, PBMCs' involvement in immune-related processes, and in the genesis of atherosclerosis, makes these cells especially appropriate for the studies of Sim and Vyt's immunomodulatory effects.

2.4. Sample RNA

Blood sample from each patient was drawn by venipuncture at each of the two baselines, and after each of the two drug therapies. PBMCs were separated on Ficoll Hypaque (Sigma). The RNA was extracted from cells using QIAGEN RNEasy kit, following the manufacturer's instructions. RNA quality and quantity were assessed using the RNA 6000 Nano LabChip kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples with poor quality RNA and/or hybridization results were removed. These criteria were defined by the ratio of 260/280, and by the lower than 35% detectable genes.

2.5. Microarray analysis

BeadChip data files were analyzed with Illumina's GenomeStudio gene expression module and R-based Bioconductor package to determine gene expression signal levels [16]. Briefly, the raw intensity of Illumina Human gene expression array was scanned and extracted using BeadScan, with the data corrected by background subtraction in GenomeStudio module. The *lumi* module in the R-based *Bioconductor* Package was used to transform the expression intensity into log 2 scale [17]. The log 2 transformed intensity data was normalized using Quantile normalization function. Map pathway analysis was conducted for the immunomodulatory genes by importing the entity lists of the transcripts for each drug treatment with *P*-values <0.05 into Genespring. The lists were compared to the pathways in BioPax 2 format from a variety of online pathway and protein–protein interaction (PPI) databases. In addition, Agilent Genespring (Agilent.com) was used as the source for online tools for cluster analyses.

2.6. RT-PCR validation

Commercially available specific primers for the representative genes were selected. RT-PCR was used to validate the expression levels of genes that showed ≥1.3 times fold changes in the microarray analysis.

For the purpose of RT-PCR, RNA was converted to cDNA via the First Strand cDNA Synthesis Kit (SABiosciencse C-08). Briefly, 240 ng of total RNA was treated with GE (Genomic DNA Elimination Buffer) and was then reverse transcribed using MMLV reverse transcriptase. The Reverse Transcription was performed in an ABI GeneAmp PCR System 2700. The Genomic Elimination took place at 37 °C for 5 min. After placing the samples on ice for 1 min, the RT mixture was added and the samples were incubated at 42 °C for 15 min. Finally the reaction was stopped by heating to 95 °C for 5 min. Real-Time PCR was performed on an ABI 7900HT thermocycler.

The cycling conditions were as follows: 95 °C for 10 min (for the HotStart DNA Polymerase), followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. At the end of each PCR run, melting curve analysis was also performed to verify the integrity and homogeneity of PCR products. The reactions were monitored using SYBR green and results were analyzed using the ABI 7900HT SDS software version 2.4. The values were normalized relative to ACTBH.

2.7. Other measurements

Lipid profiles were measured in the plasma using a Cholestech LDX apparatus (Cholestech Corporation, Hayward, CA) standardized against the laboratory assay [18]. CRP serum levels were measured by an ELISA kit (R&D Systems, Cat # DCRP00), according to the manufacturer's instructions.

2.8. Statistical analysis

We performed two separate comparisons. First we analyzed Vyt gene expression versus its baseline, and Sim gene expression versus