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Evaluation of molecular profiling platforms in clinical pharmacology

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SECTION I

General introduction

CHAPTER I

Introduction and outline of the thesis

Introduction

With the completion of the human genome project [1;2], high expectations are raised with regard to advances in our understanding of diseases and the development of better drugs to treat them. Indeed, vast quantities of data have been generated at an unmatched rate, which has provided important new insights in the pathophysiology of diseases and has led to the discovery of many promising novel drug targets [3]. Combined with the efforts from recent developments in combinatorial chemistry and high throughput screening technologies, huge libraries of lead compounds have been constructed and screened, resulting in many drug candidates that fuel the drug discovery pipelines of the pharmaceutical industry [3]. However, it is one thing to generate a plethora of drug candidates, but it is another to see more of these candidates make it through the clinical trials and ultimately reach the patient's bedside.

The bottleneck in delivering new or improved pharmacological treatments appears to have shifted from forming new hypotheses and identifying new targets (i.e. drug discovery), to choosing which candidates warrant follow-up and critical resource allocation (i.e. drug evaluation) [3]. At present, most drug candidates fail relatively late during the large-scale costly (phase III) trials because of lack of efficacy [4]. Clearly, one part of the problem is poorly predictive animal models, especially for some diseases and drugs with a novel mechanism of action [4]. Arguably, the best 'models' for drug evaluation are in fact human subjects themselves [5]. Therefore, a key element to reduce the late stage failure rates is the development and use of sensitive and selective effect parameters (i.e. 'biomarkers') that indicate *in vivo* 'target modulation' as early as possible in the clinical development of new compounds [6].

Biomarkers

Although the term biomarker historically refers to analytes in biological samples, any measurement that predicts an individual's disease state or response to a drug can be called a biomarker [7]. A biomarker is defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention' [8]. It is distinguished from a clinical end point, which is defined as 'a characteristic or variable that reflects how a patients feels, functions or survives' or a surrogate end point defined as 'a biomarker that is intended as a substitute for a clinical and point [8]. A surrogate end point is expected to predict clinical benefit (or harm, or lack of benefit or harm) based on epidemiological, therapeutic, pathophysiological or other scientific evidence [8].

To expedite the clinical drug evaluation process, there is a high demand for biomarkers that adequately, and with great specificity, indicate the presence or absence of the desired pharmacological response [6]. It has now become evident that a broader array of 'knowledge-based' (relating to the known mechanism of action), combinatorial biomarkers [9] (or biomarker *profiles*) can be used for better decision-making, i.e. to stop the development of nonviable drug candidates as early as possible and transferring the available resources to potentially more successful ones [7;10].

'Narrow' versus 'broad'

Currently, assays of drug action (i.e. biomarker assays) typically evaluate biochemical activity from a limited number of known analytes (e.g. fasting plasma glucose) or physiological parameters (e.g. blood pressure and heart rate). However, many drugs have more than one specific action and are called 'pleiotropic' [11]. Therefore, it is quite understandable that accurately matching or predicting therapeutic efficacy with a limited number of variables can be a challenge [12].

Newly developed molecular profiling approaches, defined as 'platform technologies capable of recording the entire cellular response to perturbation' [13;14], seek to provide broad information about the effects of a disease or drug into a specific 'molecular fingerprint' [15]. Biological samples, whether tissue, cells or body fluids, can be studied using a variety of molecular profiling methods, collectively called the '-omics' technologies [6]. For clarity, in this introduction the term '-omics' is used to cover the three well-established sub disciplines: transcriptomics, proteomics and metabolomics.

Approaches in molecular profiling

The cellular response to perturbation can be measured on various levels between DNA ('upstream') and metabolite ('downstream') by making use of one or more of the '-omics' sub disciplines as indicated in figure 1 (p. 201).

One of the most widely used approaches in molecular profiling is the measurement of global gene expression profiles (i.e. 'transcriptomics') using cDNA or the oligonucleotide-based microarrays (i.e. Affymetrics GeneChip®) [16]. This technology is capable of simultaneously

assessing cell or tissue gene expression levels (i.e. mRNA abundance) of more than 30,000 genes in a single experiment [17].

Proteomics entails the investigation of all proteins and their various modifications within a system, i.e. a cell, tissue, biofluid or organism. The techniques involved in proteomics (i.e. 2-D gel electrophoresis followed by mass spectrometry, liquid chromatography and immuno-assay based microarray technology (ProteinChip®)) allow the global screening of complex samples of proteins and provide a qualitative and quantitative read-out for differentially expressed proteins [18;19].

The third, most recent addition to the '-omics' profiling toolbox is called metabolomics [20]. It involves the quantification of proteins and low molecular weight molecules concentrations using i.e. NMR spectroscopy and/or liquid chromatography mass spectrometry (LC-MS) in a variety of biofluids like urine [21], blood plasma [22;23] or serum [24;25], seminal fluid [26], and spinal fluid [27].

A more detailed description of the major '-omics' sub disciplines employed in the studies comprising this thesis (i.e. transcriptomics and metabolomics) is provided in Chapter 2.

Systems biology

Comparison of the molecular profiles (i.e. gene expression or metabolic profiles in a certain tissue or biofluid) between two or more 'states' or 'classes' could indicate the global effects and affected pathways of a (pharmacological) intervention or disease state and thus constitutes a molecular 'fingerprint'.

Ultimately, the data collected from all molecular profiling sub disciplines could be integrated and also combined with data from 'clinical' measurements (i.e. blood-pressure, heart-rate, fasting plasma glucose concentrations etc.) using sophisticated analytical software, constituting the 'systems biology' approach, which aims to provide a more holistic picture of the functioning of a biological system [28]. However, the workup of these complicated computational analyses is beyond the scope of this thesis.

Type 2 Diabetes Mellitus (T2DM) as 'playground' for molecular profiling

T2DM is a multifactorial, heterogeneous disorder characterized by impaired insulin secretion on a background of insulin resistance and is typically accompanied by high triglyceride-low high density

lipoprotein-cholesterol dyslipidemia [29]. The thiazolidinedione-based glitazones [30] and fibric acid derivatives or fibrates [31], are typical drugs used in the management of T2DM and the metabolic syndrome [32].

The glitazones mainly increase insulin sensitivity and reduce glucose levels in T2DM patients by enhancing glucose metabolism in muscle tissue and decrease the rate of hepatic gluconeogenesis [33;34]. Fibrates improve the lipid profile mainly by decreasing triglyceride and increase HDL-cholesterol levels [35;36]. In addition, both classes of drugs appear to possess potent anti-inflammatory properties [37-43].

The glitazones and fibrates mediate their therapeutic effects by binding and activating the peroxisome proliferator activated receptor (PPAR), PPARγ and PPARα, respectively [44]. In addition to these two classes, a third class of PPARß/∂ agonists is currently being developed, with a potential indication in the prevention and treatment of atherosclerosis [45]. However, none of the compounds under investigation in this class have thus far attained marketing approval.

The PPARs (α,γ and ß/∂ isoforms) are members of the nuclear hormone receptor family of ligand-activated transcription factors and control the expression of many target genes regulating glucose metabolism, lipid metabolism and inflammatory processes in various tissues through at least three mechanisms [30;46-48] (figure 2; p. 201).

First, PPAR/Retinoic acid X receptor (RXR) heterodimers bind to a specific response element within the promoter sequence of their target genes. In the absence of ligands this heterodimer actively represses transcription through interactions with co-repressor complexes, a process called 'active repression' [49-52]. Second, RXR receptors can form a heterodimeric protein pair with ligand activated PPARs, and activate peroxisome proliferator-activated response elements in the promoter region of target genes, thereby increasing gene transcription, a process known as 'transactivation' [53-55]. A third mechanism of action relates to the physical interaction between ligand-activated PPAR and the binding of other pairs of nuclear transcription factors to their responsive elements in gene promoters, thereby inhibiting gene transcription, a process known as 'transrepression' [56-58].

Two PPAR isoforms are expressed at the protein level in humans, γ_1 and γ_2[59]. These differ only in that the γ_2 isoform has 30 additional amino acids at its N terminus due to differential promoter usage within the same gene and subsequent alternative RNA processing. PPARγ_2 is expressed primarily in adipose tissue [60] whereas PPARγ_1 is expressed in a broad range of tissues including heart, skeletal muscle, colon, small and large intestines, kidney, pancreas, and spleen. PPARα is highly

expressed in numerous metabolically active tissues including liver, kidney, heart, skeletal muscle, and brown fat [61;62] and it is also present in mononuclear cells [63], vascular endothelial cells [64], and vascular smooth muscle cells [37]. A third isoform PPARß/∂ (and the last of the PPARs to be identified [65]) is ubiquitously expressed in human organs and has been proposed to act as 'lipid sensor' [66].

The notion that PPAR agonists are known to regulate gene expression and have well documented effects on biochemical markers in human plasma [35;36;67;68], makes these drugs ideal for the evaluation of new molecular profiling technologies, which could serve as important tools for the identification of 'mechanism-based' efficacy biomarker patterns or molecular 'fingerprints'. Eventually these 'fingerprints' could be used to support and expedite the clinical development of new drug candidates. For this evaluation, rosiglitazone and ciprofibrate were selected as 'prototype' drugs to generate a putative PPARγ and PPARα class-specific molecular fingerprint, respectively.

Furthermore, we chose to study T2DM patients and healthy volunteers (HVs) in parallel. This allowed us to assess if the anticipated molecular 'treatment fingerprints' could also be identified in more easily studied and recruited HVs. In addition, this provided an opportunity to explore the baseline differences in molecular profile between the two study groups and thus identify a putative T2DM 'disease fingerprint'.

The outstanding question is whether any one or both of these new molecular profiling technologies – and pillars of 'systems biology' – are sufficiently robust to generate reliable results and thus allow the potential future application of these 'mechanism-based' molecular biomarkers to early clinical development 'proof of concept' studies with novel (PPAR agonist) drug-candidates.

Outline of the thesis

This thesis is divided in five major sections. After a brief introduction (Chapter 1), the first section contains a more detailed description of the molecular profiling platforms and some background on the biostatistical analyses employed in the studies of this thesis (Chapter 2).

The second section describes the results of the analyses performed to show the baseline characteristics of the two studygroups and demonstrates the treatment response of the two PPAR(γ and α) 'prototype' drugs in T2DM patients and HVs by assessing a set of both 'traditional' and 'non-traditional' biochemical markers (Chapter 3 and 4).

The third section contains a description of the studies in which we evaluated the feasibility and methods of transcriptional profiling (using spotted cDNA microarray technology) for the assessment of the pharmacological effects of PPAR agonists in their target tissues. In addition, the baseline differences in gene expression profile between the T2DM patients and HVs were explored to identify putative disease related genes within certain biological functions / pathways.

To evaluate the feasibility of human tissue gene expression profiling in a clinical pharmacology setting, we first performed a pilot study in which the tolerability of minimally invasive human skeletal muscle and adipose tissue biopsy procedures were evaluated (Chapter 5). In addition, we evaluated the performance of our RNA extraction and cDNA microarray hybridization methods, data normalization procedures as well as methods to assess data reproducibility and outlier detection.

Chapter 6 describes the assessment of the pharmacological effects of rosiglitazone (PPARγ agonist) on global gene expression profiles in skeletal muscle, adipose tissue and peripheral blood leukocytes of T2DM and HVs using cDNA microarrays in order to identify a molecular 'treatment fingerprint'. In addition, this chapter describes the exploration of the baseline differences in gene expression profile between T2DM patients and HVs to identify a putative 'disease fingerprint'.

Analogous to Chapter 6, Chapter 7 describes the evaluation of the pharmacological effects of ciprofibrate on global gene expression profiles and exploration of the baseline differences in gene expression profile between T2DM patients and HVs. In addition, the chapter describes the comparison of the putative disease related gene expression profiles between the two studies in order to explore the 'external' reproducibility of these profiles and to identify potentially 'enriched' (overlapping) T2DM-related biological functions and pathways within these profiles.

Section four describes the evaluation of NMR spectroscopy-based metabolite profiling ('metabolomics') as tool for the assessment of pharmacological (PPAR) effects and exploration of the baseline differences in urine and plasma metabolite profiles between T2DM patients and HVs. In Chapter 8 we describe the pharmacological effects of rosiglitazone on global metabolite profiles in urine and plasma as well as the exploration of baseline differences between T2DM patients and HVs. Subsequently, in Chapter 9 we describe the pharmacological effects of ciprofibrate on global metabolite profiles in plasma as well as the

exploration of baseline differences between T2DM patients and HVs. Section five contains the summary (Chapter 10), general discussion and conclusions of the thesis (Chapter 11) as well as the introduction, results and conclusions of the thesis in Dutch (Chapter 12).

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