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Prediction of adverse events by in vivo gene expression profiling exemplified for phytopharmaceuticals containing salicylates and the antidepressant imipramine

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This contribution is dedicated in memoriam to Prof. Dr. Hilke Winterhoff, Institute for Pharmacology and Toxicology, Westfälische Wilhelms-University, Münster, Germany who initiated this work and passed away on May 9, 2010.

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ABSTRACT

Background and objective: Gene expression profiles of Sprague-Dawley (SD) rats treated with a standardized willow bark extract (WB), its salicin rich ethanol fraction (EtOH-FR) or the tricyclic antidepressant imipramine were evaluated for their potential to induce adverse events. Treatments had shown antidepressant-like effects.
Methods: Gene expression profiles (Agilent Whole Genome Array, $n = 4$/group) obtained from the peripheral blood of male SD rats treated with WB (STW 33-I), EtOH-FR (30 mg/kg bw) or imipramine (20 mg/kg bw) were analysed comparatively by the Ingenuity Systems Programme, which allows to conduct model calculations of thresholds for theoretical potential adverse events (AE).
Results: The number of genes regulated by the three treatments were 1673 (WB), 117 (EtOH-FR) and 1733 (imipramine). The three treatments related to 47 disease clusters. The WB extract reached the threshold for a potential AE in one disease cluster (cardiac hypertrophy), whereas the EtOH-FR exceeded the threshold in 5 disease clusters (cardiac arteriopathy and stenosis, glomerular injury, pulmonary hypertension, alkaline phosphatase levels ⇑). Imipramine treatment hit 13 disease clusters: tachycardia, palpitation, myocardial infarction, arrhythmias, heart block, precipitation of congestive heart failure; urinary retention, altered liver functions. Those correspond to known potential adverse events. Glomerular injury and altered liver functions are part of the side effect profile of salicylic acid derivatives in agreement with the findings for the salicin rich EtOH-FR.
Conclusion: There is no linear relationship between the number of constituents of a drug (preparation) and the number of different targets hit in a biological system on the gene expression level. Therefore, the number of genetic targets in a biological system does not necessarily increase with the complexity of the treatment corresponding to the non-linear behaviour of biological systems. Regarding gene expression levels AE of single treatments are not necessarily additive in combination treatments.

The applied method appears to be an interesting screening tool for the prediction of potential AE. The phenomena that imipramine crossed the potential threshold for AEs several times whereas the WB extract did reach the threshold level only once, however not backed by clinical data for this AE, deserves to be further investigated. It questions the commonly assumed principle that substances with low number or without AE will have a poor efficacy.

Abbreviations: 4EBP1, eukaryotic translation initiation factor 4E binding protein 1; ADRA1D, adrenergic α-1D-receptor; AE, adverse events; ACE, angiotensin I converting enzyme (peptidyl-dipeptidase A) 1; SD, Sprague-Dawley; DAG, diacylglycerol; DMARD, disease modifying anti-rheumatic drug; ECM, extra cellular matrix; EDNRA, endothelin receptor; eIF4s, mRNA binding translation factors; EPO, erythropoietin; ER, estrogen receptor; EtOH-FR, ethanol fraction; FES, Agilent Feature Extraction software; FST, Porsolt Swimming Test; G protein, guanine-nucleotide-binding protein; HIF, hypoxia inducible factor-1; IP3, phosphatidyl-inositol-1,4,5 triphosphate; IPA, Ingenuity Pathways Analysis; JNK, c-Jun N-terminal kinase; MS, multiple sclerosis; MTX, methotrexate; NOS1, nitric oxide synthase 1; PAD, peripheral arterial disease; PDGF, platelet-derived growth factor beta; RXR, retinoid x receptor; TIMP3, tissue inhibitor of metalloproteinase 3; VDR, vitamin D (1,25-dihydroxyvitamin D3) receptor; VEGF, vascular endothelial growth factor; WB, willow bark extract.

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Introduction

The correct prediction of adverse events (AE) of synthetic drugs, phytopharmaceuticals or their combinations is essential for drug development as well as for drugs already in use. In the context of toxicity testing the report by the National Research Council of the US National Academy of Science, *Toxicity Testing in the 21st Century* has prompted a discussion to base the assessments on "mechanisms and toxicant modes of action" and to identify "pathways of toxicity" (Hartung and McBride 2011). Hartung and McBride (2011) proposed very recently the "Mapping of the human Toxome" as the basis for a new testing approach facilitating the identification of non-toxicity. The OECD coined the term "adverse outcome pathways" (Ankley et al. 2010; Hartung and McBride 2011) to be investigated. We proposed earlier as a rather simple (and first step) approach to apply ready-to-use topic defined Toxgene microarrays as pre-screening tools for phytopharmaceuticals (Ulrich-Merzenich et al., 2009b).

In the present study we used whole genome gene expression profiles, obtained from rats which had shown a positive response in a standard research model for depression – the Porsolt Swimming Test (FST) – and had been treated with either the tricyclic antidepressant imipramine, the standardized willow bark extract STW-33-I (WB), or its salicin rich ethanol fraction (EtOH-FR) (Ulrich-Merzenich et al., 2012) for a theoretical analysis (Ingenuity) for the prediction of AE. Detailed results on the treatment-specific individual gene regulation are reported under Ulrich-Merzenich et al. (2012).

Here, based on the expression profiles, the prime signalling cascades activated by the different treatments are described and the treatment specific expression profiles are compared with published data of reports about the relation between the modulation of genes and potentially occurring adverse events. For this purpose the Ingenuity programme offers a so-called "toxic endpoint analysis". In this analysis well studied groups of genes/molecules which are known to participate in clinical pathology (histopathology or clinical chemistry) and to lead to toxicological events or processes within specific tissues are searched for in the submitted gene expression profiles.

The essential parameters in the analysis is the negative logarithm of the *p*-value (calculated with a right tailed Fisher's exact test) on networks (in the comparison of regulated genes in the different treatments) The higher the score, the less likely the molecules/genes (regarded as tox molecules) within the network are associated due to chance. We finally examined whether the potential targets derived by this evaluation method correspond with the known adverse events of the applied treatments.

Willow bark as originator plant for the development of the non-steroidal anti-inflammatory drugs and its designated principle "salicin" as well as the antidepressant imipramine are known drugs with well documented AE profiles, especially in the case of imipramine. An intensive discussion of their mode of action in the experiments performed here on different genes are reported under Ulrich-Merzenich et al. (2012). This work will concentrate on the analysis of the activated signal cascades, the potential AE and the corresponding genes.

Materials and methods

The dried willow bark preparation STW 33-I (WB) was obtained from Steigerwald Arzneimittelwerk GmbH, Darmstadt, Germany. Extract was prepared from willow bark according to PhEur. 6.1, with a DEV_{nativ} of 16–23:1, total salicin content 23–26% (m/m). Imipramine hydrochloride was obtained from Sigma (Deisenhofen, Germany).

Preparation and characterisation of the tested fractions

The investigated fraction was prepared as described in Freischmidt et al. (2011a) by application of subsequent partition steps using, among other solvents, also ethanol (EtOH-FR). A quantitative and qualitative determination of different classes of compounds in the WB and the resulting fractions was done. The salicin and salicyl alcohol content was determined according Ph.Eur 6.4, total polyphenol, tannin and rest phenol content was quantified according to Glasl (1983). As the flavonoid spectrum of willow bark mainly consists of flavanone and chalcones glycosides the common PhEur methods for determination of overall flavonoid content is not applicable. Thus, it was determined according a newly developed method (Freischmidt et al., 2011b).

The WB is rich in salicin, salicin derivates and polyphenols; whereas the EtOH-FR contains a high amount of salicin and salicin derivates while having comparatively low polyphenol content as described earlier (Ulrich-Merzenich et al., 2012).

Animals

Male Sprague-Dawley (SD) rats (150–170 g, Charles River Laboratories, Sulzfeld, Germany) were housed in groups of two and kept in conditioned rooms (24 ± 1 °C, light–dark cycle 12/12 h). Animals had free access to food (Altromin® 1324, Altromin, Lage, Germany) and tap water. The procedures used comply with the European Community's Council Directive of 24 November 1986 (86/609/EEC) and were officially approved by the local committee on animal care (Regierungspräsident Münster, AC/2004).

Test substances

Animals ($n = 12$ per group) received the test solutions (WB, its EtOH-FR, or imipramine, suspended in water, 10 ml/kg b.w.) p. o. once daily as described earlier (Ulrich-Merzenich et al., 2012). The solvent was used as negative control.

Gene microarrays

Blood samples (3 ml) of treated and untreated rats were collected in PAX-gene collection tubes (Preanalytix) 2 h after the performance of the Porsolt Swimming Test (FST). RNA was isolated by Pax Gene Blood RNA Kit (Qiagen) and the gene modulation was determined in four animals per group. The RNA-Integrity numbers (Agilent 2100 Bioanalyzer) of the isolated RNAs were between 7.3 and 8.8. Only RNA of treatment groups which showed significant responses in the FST compared to the untreated group were selected for detailed microarray analysis. For analysis single colour hybridization of the rat RNA on the Rat Agilent Whole Genome Oligo Micorarrays (41013 genes) after T7 RNA amplification was performed (Miltenyi Biotec, Bergisch Gladbach, Germany). The Agilent Feature Extraction software (FES) was used to read out and process the microarray image files. For the determination of the differential gene expression FES derived output data files were further analysed using the Rosetta Resolver® gene expression data analysis system (Rosetta Biosoftware). The background corrected intensity data were used for the calculation of the ratios control/experimental sample. The ratios were computed using a common "artificial reference" (4 control samples combined). This common reference was used as baseline for all samples. A global correlation analysis of all ratio data was performed. Data sets were filtered in order to remove genes which are not differentially regulated in any comparison.