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Invited Perspective

Correlation and cluster analysis of immunomodulatory drugs based on cytokine profiles

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ABSTRACT

Drug discovery is a constant struggle to overcome hurdles posed by the complexity of biological systems. One of these hurdles is to find and understand the molecular target and the biological mechanism of action. Although the molecular target has been determined, the true biological effect may be unforeseen also for well-established drugs. Hence, there is a need for novel ways to increase the knowledge of the biological effects of drugs in the developmental process. In this study, we have determined cytokine profiles for 26 non-biological immunomodulatory drugs or drug candidates and used these profiles to cluster the compounds according to their effect in a preclinical *ex vivo* culture model of arthritis. This allows for prediction of functions and drug target of a novel drug candidate based on profiles obtained in this study. Results from the study showed that the JAK inhibitors tofacitinib and ruxolitinib formed a robust cluster and were found to have a distinct cytokine profile compared to the other drugs. Another robust cluster included the calcineurin inhibitors cyclosporine A and tacrolimus and the protein kinase inhibitors fostamatinib disodium and sotrastaurin acetate, which caused a strong overall inhibition of the cytokine production. The results of this methodology indicate that cytokine profiles can be used to provide a fingerprint-like identification of a drug as a tool to benchmark novel drugs and to improve descriptions of mode of action.

1. Introduction

One of the most important steps in drug discovery is to identify and validate the target of a new compound. Identifying the target as well as description of possible alternative targets provide valuable information since the therapeutic effect and possible side effects of the drug candidate can be predicted in the early phase [1]. Even in the present days of big data and rational drug design, there is a constant need of deeper knowledge of the biological effects of drugs and drug candidates. In addition, there is a need for tools to deal with the complex drug mechanism as well as identification of valuable biomarkers and clinical endpoint phenotypes for evaluation of drug effects. This is especially true for compounds developed through phenotypical assays, but also for compounds where the molecular target is known. The complexity of biological systems with interconnected pathways, feedback loops, off-target effects, and individual variations often convolutes the active mechanisms. Also, the importance of polypharmacology is increasingly acknowledged adding another level of complexity [2].

One way to increase the knowledge gained from data is to extract patterns and similarities from a multitude of inputs and correlate different compounds and disease mechanisms such as exemplified by the "connectivity map" created by Lamb et al. [3]. The connectivity map is based on gene expression profiles from cultured human cells treated by bioactive compounds to enable the functional connections between drugs, genes and diseases. By using a wide variety of small-molecule perturbagens, they created a system capable of connecting biological processes and therapeutics. This system could then be used to find compounds with common mechanism of action in order to find target pathways and to identify potential therapeutic opportunities. Recently, the connectivity map was vastly extended to include over a million profiles, which could be used to predict mechanism of action of small molecules [4]. With these promising results, we set out to create a more focused system, based on immunologically active compounds and cytokine profiles as a measure of immunomodulation. Our approach was to

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use cytokine pattern profiling in primary cells extracted from preclinical rat models of autoimmune inflammation [5]. Using *ex vivo* cultures as a model system of inflammation provides the advantage of mimicking the *in vivo* system while excluding complex factors such as drug formulation and bioavailability [6].

Characterization of the immune regulation can be accomplished by measuring molecular biomarkers such as cytokines, chemokines, large scale proteomics [7], gene expression profiling [8] or more recently by micro RNA profiling [8,9]. As a medium scale system to depicture the immune regulating complexity and network we have chosen profiling of cytokine response in our preclinical model. Cytokines are central molecules that regulate the immune system and are usually classified into groups based on their function or because they are produced by the same immune cell. The pro-inflammatory cytokines, which induce inflammation, include interleukine-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNF-α) [10,11]. Cytokines are also highly involved in the development and activation of the adaptive immunity, and especially in T-cell development. IL-2 stimulates the activation of T-cells [10], which develop into either Th1 or Th2 cells. Th1 cells are characterized by their production of IL-2 and IFN-γ whereas Th2 cells produce the cytokines IL-4, IL-5 and IL-13 [12,13]. In addition, Th17 cells are defined by their production of IL-17, which is a cytokine that induces inflammation [14]. Many chemokines, such as RANTES, MIP-3α, GRO/KC are examples of molecules that are involved in inflammation by recruiting immune cells to the infected site [11]. Also, cytokines such as IL-7, erythropoietin (EPO) and vascular endothelial growth factor (VEGF) are important growth factors for the immune system [15–17].

The immune system with its specific cytokine pathways is obviously a very important target for disease interventions. Immunomodulation includes both activating and suppressing immunotherapies and is achieved using both cells, biological agents and small molecules. Activating immunotherapies are increasingly used, especially with the novel cell therapies for treating cancer. Also antibodies and cytokines as well as small molecules are used to activate the immune response to restore it in patients with compromised immunity. Immunosuppressive drugs are used to suppress the immune response, to treat e.g. allergic, inflammatory and autoimmune diseases or to prevent rejection after organ transplantation [18–20]. Immunosuppression involves reduction of the activation and efficiency of the immune systems by suppressing mainly signaling pathways of the immune cells. A range of different drugs have been developed to inhibit the immune system. Four interesting groups can be devised based on their mode of action, namely immunophilin targeting drugs, glucocorticoids, protein kinase inhibitors and antibodies [18,19]. Immunophilin targeting drugs include the calcineurin inhibitors tacrolimus and cyclosporine A as well as the mammalian targets of rapamycin (mTOR) inhibitors such as sirolimus and everolimus [18]. The glucocorticoids are steroids that bind to the glucocorticoid receptor and thereby up-regulate the expression of several anti-inflammatory proteins, leading to both immunosuppressive and anti-inflammatory effects [18]. Examples of glucocorticoids include the drugs prednisolone, dexamethasone and fluticasone propionate. The protein kinase inhibitors block the activity of the protein kinase enzymes, which are central enzymes for many signaling pathways in immune cells. The protein kinase inhibitors include, for example, the janus kinase (JAK) inhibitors tofacitinib and ruxolitinib as well as the tyrosine kinase inhibitors nilotinib and fostamatinib disodium [19,20]. Antibodies used in immunosuppression are often targeting specific molecules of the signaling system, such as the IL-2 receptor, CD3 and TNF-α, which thereby downregulating the immune response [18].

Given the fact that immunosuppressive drugs have distinct targets it is likely that different groups of drugs will modulate the cytokine production differently. At the same time, its complexity, feedback mechanisms and redundancy makes it difficult to assess a distinct mechanism of action also for well-characterized drugs without engaging and analyzing the whole immune system reaction. However, by assessing the cytokine profile of a specific condition such as a drug treatment or a disease state, a lot can be learned about which immune pathways that are affected. This allows knowledge to be made about the disease mechanism and drug targets, similarly to the gene expression profiles in the connectivity map. The aim of this study was to analyze how different drugs affect the cytokine production and to compare the drugs based on their cytokine profiles. Data obtained from this study could then be used to predict molecular targets and mode of action of immunomodulatory drug candidates by comparing their cytokine profiles to the profiles generated by the well-characterized immunomodulatory drugs included in this study.

2. Materials and methods

2.1. Animals

Rats, Dark Agouti (DA) (Janvier, Europe), were kept in animal facilities in a climate-controlled environment with 12 h light/dark cycles, housed in polystyrene cages containing wood shavings and fed standard rodent chow and water *ad libitum* in the animal house of Medicon Village, Lund, Sweden. The rats were found to be free from common pathogens. The experiments were approved by the local ethical committee (Malmö/Lund, Sweden, M167-12).

2.2. Cell preparation and activation

Female DA rats, 8–10 weeks of age, were injected with 500 μl of the adjuvant pristane (Acros Organics, Geel, Belgium) s.c. at the base of the tail at day 0 in order to pre-stimulate an immune response [21]. At day 14, at the onset of arthritis, rats were sacrificed and spleens were collected from 9 rats. Single cell suspensions were prepared by passing the cells through a 40 μm cell strainer (BD Falcon, San Jose, CA, USA) with a piston from a 5 ml syringe. Red blood cells were lysed in BD pharmlyse buffer and the remaining cells were washed with HBSS [21]. Cells were diluted to 4.5×10^6 cells per ml of RPMI medium containing 3 μg/ml of ConA (Sigma) and selected drug (see below). Cells were incubated in a 96 well plate in a standard incubator (37 °C and 5% CO_2) for 44 h. Supernatants were harvested from cell culture plates and frozen at −20 °C and stored at −80 °C until assayed (supernatant was analyzed within five months from preparation). Drugs were analyzed on nine separate biological replicates.

2.3. Drugs

The tested drugs were diluted to 10 mM stock solutions in DMSO (stored in dark at room temperature for long time use) or in mqH_2O (prepared fresh prior to assay). The final concentrations of the drugs were determined as the maximal concentration that did not induce cell apoptosis but still caused decrease of IFN-γ or IL-2 levels according to dose response curves measured by ELISA during prior experimental calibration studies (data not shown). The final concentration of DMSO in analyzed samples was 0.5%. The following compounds and their concentrations were used in the cell cultures: apremilast (Selleck Chemicals; 0.125 μM), apilimod mesylate (Axon MedChem; 125 nM), astaxanthin (SantaCruz BT; 50 μM), bardoxolone methyl (Toronto Research Chemicals; 6.25 nM), bortezomib (Selleck Chemicals; 6.25 nM), cyclosporine A (Sigma; 125 nM), dexamethasone (Sigma; 125 nM), dimethyl fumarate (Sigma; 125 nM), everolimus (Selleck Chemicals; 2.5 μM),

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