MDR—ABC transporter activity in normal population and in rheumatoid arthritis patients. A predictive tool of biological therapeutic response.

Ph.D Thesis

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1. Introduction

In the past 10 years, a paradigm shift has taken place in understanding the clinical significance of mechanism of action of multidrug resistance (MDR) transporters and their function as potential biomarkers.

Earlier, it was assumed that the mechanism behind impaired therapeutic efficacy of certain drugs and multidrug resistance - ATP-binding cassette (MDR—ABC) transporter activity is solely associated with the drug efflux function. In addition to drug transport more and more evidence emerged that the transporters have an important role as regulators of immune response and inflammation representing the most important efflux mechanism for several inflammatory signaling molecules, such as platelet-activating factor (PAF), phingosine-1-phosphate (S1P), and eicosanoids (prostanoids and leukotrienes) which are among the mediators of chronic inflammation. MDR—ABC transporters may also modulate cellular redox homeostasis. In addition, several studies have showed the clinical significance of MDR MDR—ABC transporters as prognostic and/or predictive marker in immunosuppressive therapies for active rheumatoid arthritis (RA) using methotrexate, other synthetic disease-modifying anti-rheumatic drugs (sDMARDs) or biological DMARDs.

According to the new concept MDR—ABC transporters are biomarkers. Their role in the immune processes and MDR is a rapidly developing field and it will be likely evaluated as part of a complex panel of biomarkers for prognostic scoring, for monitoring disease activity or to predict the responsiveness to certain medications (e.g. immunosuppressive treatments or chemotherapy in malignancies). However, translation of MDR—ABC transporter activity into clinical decisions and treatment regimen requires well defined normal reference and pathological activity values.

This thesis explores how the new scientific findings might establish MDR—ABC transporters as predictive biomarkers in rheumatoid arthritis (RA).

Focusing on the importance of the multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) transporters in rheumatoid arthritis.

1.1. Rheumatoid arthritis

Rheumatoid arthritis is one of the most common chronic inflammatory autoimmune diseases affecting about 0.5–1% of the world population.

RA is more prevalent in female and it is often occurred at the 5th decade of the life. The disease is characterized by the overactivation of the immune system and progressive joint destructions. Persistent synovial inflammation finally results in joint and bone malformation which causes disability, that drastically cuts down the patient's quality of life. As a consequence of widespread inflammation the function of other organs and tissues such as the heart, the lung and the blood vessels are impaired as well.

In the last 20 years, drastic improvements in RA treatment have been developed by applying a wide variety of new synthetic and biologic DMARDs such as anti-tumour necrosis factor (TNF) antibodies (e.g adalimumab, certolizumab pegol, etanercept) and T-cell inhibitors (e.g abatacept). Importantly, early intervention with DMARDs maintains long-term functional activity of affected joints, by preventing them from tissue damage.

Several studies examined the possible role of MDR proteins in autoimmune disorders e.g. RA and focused on the correlation with disease activity, therapy responsiveness and progression (outcome). MDR expression may correlate with RA disease activity, as well as with responses to methotrexate and other DMARD treatment. There have been very little data available on the possible association between MDR activity and responses to biologics.

1.2. Need for biomarkers in RA disease management

Disease management of RA is a costly and challenging exercise for a notable part of patients.

In 2018 in the USA the average direct cost of treatment for RA is 12.509 USD/year for synthetic DMARDs and f 36.503 USD/ year for biologic DMARDs. Methotrexate, other synthetic DMARDs and numerous new biological DMARDs are used in monotherapy or in combination. Despite the new generations of drugs, there still remains a large unmet patient need in the treatment of RA.

RA is a disease with a highly variable prognosis, quickly leading to disability in many cases.

Despite of all the recent development in RA treatment all current therapies have poor response rates (methotrexate: 50%, sDMARDS: 63%, bDMARDS: 50%) and serious side effects, particularly if the first treatment is delayed, and there is no predictive biomarker available for drug efficiency to choose between therapies upfront.

Also there is no good disease activity marker available on the market to show drug response and change the therapy early on.

2. Aims

The importance of MDR—ABC transporters in RA is well published. However, that MDR—ABC transporter activity may be of predictive value for biological therapies has not been established.

The aim of this project was to gain an understanding of the characteristics of MDR—ABC transporter activity in normal population and in RA patients. And establish it as a predictive biomarker in biological therapy.

We investigated

1. The activity of three clinically relevant transporter, MDR1, MRP1 and BCRP in CD3+ lymphocytes from

healthy volunteers in order to describe normal reference values.

2. The effects of gender and age on transporter activity reference values.
3. Is there a change in MDR1, MRP1 and BCRP activity in CD3+ and CD19+ lymphocytes from RA patients during biological therapy?
4. How to utilize the different transporter activity characteristics as a predictive tool of biological therapeutic response in patients before as well as 4 to 6 and 12 weeks after the initiation of biological therapy.

3. Materials and methods

Two clinical studies are discussed in this thesis.

In the first, normal reference range study 120 healthy volunteers were included in order to describe normal reference values of the activity of MDR—ABC transporters on CD3+ peripheral blood mononuclear cells (PBMC). The effects of gender and age were also determined.

Using the normal reference values, in a second study we determined the predictive value of MDR1, MRP1 and BCRP activity measurement for therapeutic response in 39 RA patients. Measured on CD3+ and CD19+ PBMCs before as

well as 4 to 6 and 12 weeks after the initiation of biological therapy. 35 Healthy volunteers were also included as control.

The MultidrugQuant™ Kit was used for measurements. In this flow cytometric assay, fluorescent reporter substrates (calcein AM for MDR1 and MRP1 and mitoxantrone for BCRP, respectively) are trapped in the cytoplasm and pumped out by MDR proteins depending on the presence or absence of specific inhibitors (verapamil for MDR1 and MRP1, indomethacin for MRP1 and KO134 for BCRP, respectively), allowing for quantitative, standardized assessment of MDR activity factor (MAF). Activities of multidrug transporters are reflected by the difference between the amount of calcein/mitoxantrone accumulated in the presence or absence of the selective inhibitor(s). When calculating the MAF values, this accumulation difference is normalized to the dye uptake measured in the presence of the inhibitor. Thus, the result of the test becomes independent from factors influencing the cellular accumulation of calcein other than the activity of the multidrug transporters. MAFC is a composite activity value for the MDR1 and MRP1 transporters.

Informed consent was obtained from all subjects, and our study was approved by an independent ethical committee of the institutions.

4. Results

Our results from the normal reference values study indicate that MAF value of MRP1 is 2.5 [0.0 – 12.5] (median [2.5 – 97.5 percentiles]) and are independent from age. MAF value of BCRP is 3.4 [0.0 – 22.0] and is also independent from age. On the other hand, MAFC and MAF of MDR1 show negative correlation with the age of the studied subjects. MAF value of MDR1 is 12.9 [0.0 – 25.7] and that of MAFC is 16.5 [0.0 – 32.0]. MDR1 activity greatly contributes to the MAFC value and is therefore likely to be accountable for its similar correlation with age.

No difference was detected in any of the four MAF values between men and women. Gender does not affect the presence or lack of correlation between MAF values and age.

In the predictive biomarker assessment study disease activity score 28 joint count (DAS28) values decreased upon treatment in responders in contrast to nonresponders. Of note, initial DAS28 values were higher in the responder group compared to nonnresponders. Neither differences were observed in C-reactive protein (CRP) values between the two group, nor changes in CRP were demonstrated upon treatment. MAF of MRP1 in CD3+ cells was higher at 12 weeks in nonresponders compared to controls. No other statistically significant

difference was noted in MAF values between controls and RA patients. Control values were within the reference range established in our earlier study. At the start of therapy, MAFC and MAFMDR values of CD3+ cells were higher in nonresponders compared to responders. At 6 weeks, MAFC, MAFMRP and MAFMDR values of CD3+ cells as well as MAFMRP values of CD19+ cells were higher in nonresponders compared to responders. No significant changes were demonstrated in MAF values in the respective RA patient groups with the progress of treatment. No difference was demonstrated in MAFBCRP values in CD3+ or CD19+ cells between responders and nonresponders. Receiver operating characteristic (ROC) analysis was performed to evaluate the predictive value of MAF for response to treatment in RA patients at the start of biological therapy and at 6 week. Cut-off thresholds were calculated for MAF values with ROCs of adequate probability (p) and area under the curve (AUC) values. Patients with MAF values above the respective cut-off thresholds are likely to be nonresponders to treatment.

Cut-off values on CD3+ cells:
0 week MAFC = 21.3 ($p = 0.043$, AUC = 0.68)
6 week MAFC = 20.3 ($p = 0.033$, AUC = 0.72.)

6 week MAFMRP = 6.0 (p = 0.049, AUC = 0.69)

6 week MAFMDR = 13.9 (p = 0.048, AUC = 0.70)

5. Novel results of the thesis

I. Reference MAF values were established on CD3+ lymphocytes in a normal population, providing a baseline to compare the pathological transporter activity in diseases.

II. MAF MDR1, and its derivative MAFC decrease with age. It must be considered when MDR1 substrate drugs are administered.

III. No gender dependence was found for any MAF values.

IV. At baseline MAFC and MAFMDR values, on CD3+ cells, are higher in nonresponders to anti rheumatic biological therapy compared to responders. It may be of predictive value before the initiation of biological treatment.

V. During the biological treatment at 4 to 6 weeks MAFC, MAF MRP and MAF MDR values of CD3+ cells and MAF MRP values of CD19+ were higher in nonresponders to anti rheumatic biological therapy compared to responders. It may be of predictive value during biological treatment.

6. Summary

In conclusion, our results indicate that the determination of MAFC values in CD3+ cells of RA patients may be of predictive value prior to the initiation of biological therapy to establish whether the patient will demonstrate sufficient therapeutic response. Measuring MAFC, MAFMRP and MAFMDR values in CD3+ cells at 4 to 6 weeks after the start of treatment further improves the accuracy of prediction as to whether adequate therapeutic response may be expected.

The determination of the functional activity of MDR-ABC transporters is achievable using a flow cytometry based standardized method. Having established the normal range of MAF values of a healthy population, and determined the cut-off thresholds for MAF values between biological therapy responders and nonresponders our results allow for the development of novel flow cytometry based p algorhytms in rheumatoid arthritis.

7. Articles related to the subject of the thesis

I. Szerémy P, Tauberné Jakab K, Baráth S, Apjok A, Filkor K, Holló Z, Márki- Zay J, Kappelmayer J, Sipka S, Krajcsi P, Toldi G. Determination of Reference Values of MDR-ABC Transporter Activities in CD3+ Lymphocytes of Healthy Volunteers Using a Flow Cytometry Based Method. Cytometry B Clin Cytom. 2019 Nov;96(6):469-474. doi: 10.1002/cyto.b.21729.

II. Toldi G, Batel P, Baráth S, Szerémy P, Apjok A, Filkor K, Szántó S, Szűcs G, Szamosi S, Häupl T, Grützkau A, Szekanecz Z. Peripheral Lymphocyte Multidrug Resistance Activity as a Predictive Tool of Biological Therapeutic Response in Rheumatoid Arthritis. J Rheumatol. 2019 Jun;46(6):572-578. doi: 10.3899/jrheum.180793.

Patent related to the subject of the thesis:

III. Toldi G, Filkor K, Szerémy P, Apjok A, Szekanecz Z Assessing Responsiveness of Rheumatoid Arthritis Patients to Biological Treatment.
Patent No: EP3570028A1 Priority Data: 15.05.2018

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Co-author certification

I, myself as a corresponding author of the following publication(s) declare that the authors have no conflict of interest, and **Péter Gábor Szerémy** Ph.D. candidate had significant contribution to the jointly published research(es). The results discussed in her thesis were not used and not intended to be used in any other qualification process for obtaining a PhD degree.

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The publication(s) relevant to the applicant's thesis:

Toldi G, Batel P, Baráth S, Szerémy P, Apjok A, Filkor K, Szántó S, Szűcs G, Szamosi S, Häupl T, Grützkau A, Szekanecz Z. Peripheral Lymphocyte Multidrug Resistance Activity as a Predictive Tool of Biological Therapeutic Response in Rheumatoid Arthritis. J Rheumatol. 2019 Jun;46(6):572-578. doi: 10.3899/jrheum.180793. Epub 2019 Feb 1. PMID: 30709954.

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