Development of pharmacoproteomic platforms for monitoring changes in the thrombin mediated signalling and aggregation of human platelets treated with direct thrombin inhibitors

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ABSTRACT

Thrombin is a serine-protease known to induce the activation of platelets to aggregate by binding to and cleaving the extracellular N-terminal domains of protease-activated receptors 1 and 4 (PAR1 and PAR4). Dabigatran is an oral anticoagulant and a reversible direct inhibitor of thrombin (DTI), thereby inactivating both fibrin-bound and free (ie, free) thrombin. Recently, it has been shown that dabigatran can inhibit platelet aggregations through a direct effect on platelet thrombin receptors (PAR-4), which is mainly correlated with a reduced expression of GPIb/IIa, CD63, and P-selectin on platelets after dabigatran treatment (1).

We further hypothesize that a system biology approach coupled with global proteomics analysis of thrombin activated human platelets treated with dabigatran would reveal the interlinked kinase (ILK) and integrin mediated signaling as major significantly down-regulated pathways by the DTI dabigatran.

To test our hypothesis, we developed a pharmacoproteomics platform that monitor changes in the protein expression profiles of platelets, purified from healthy human donors and ex-vivo activated in presence or absence of the dabigatran.

The pharmacoproteins platform employed the nanolC-ESI MS/MS sequencing of tryptic/Glu-C/Lys-C generated peptides from platelets releasates, as well as a Q-Exactive quadrupole orbitrap mass spectrometer coupled with the Q-Exactive Orbitrap (LFC) module provided by PEAKS X (Bioinformatics Solutions Inc.). The proteomic analysis retrieved about 300 proteins from platelets treated with dabigatran as compared with 500 proteins from the platelets control, untreated (FDR <1.0% for proteins and <5.0% for peptides). The quantitative analysis of the biochemical pathways was accomplished with ingenuity pathway analysis (IPA, Ingenuity Systems) using the protein M1 and M2 exclusive spectra counts ratios extracted from LFQ analyses. The bioinformatics analysis predicted that many proteins involved in the actin-mediated cell signaling and movement pathways, including rhoA, rhoGDI, clathrin mediated-endocytosis, and ILK and integrin signaling pathways, were at least two-fold statistically significant down-regulated (p<0.05) in the dabigatran-treated platelets. Remarkably, biomarkers from the interlinked mediated signaling pathways (such as of GPIb/IIa) were validated to be down-regulated by the dabigatran treatment of thrombin activated platelets. In addition, the pharmacoproteomic platform was further validated with novel DTI receptor lead-ins in plasma showed significantly improved thrombin-induced platelet aggregation at concentrations of 0.8-2.0 µM. These data further validate for the use of the LFC proteomics profiling as a reliable assay for monitoring the efficacy of selected drug treatment during ACS management.

RESULTS

Working Hypothesis

- It has been shown recently that dabigatran can inhibit platelet aggregation through a direct effect on platelet thrombin receptors (PAR-4), which is mainly correlated with a reduced expression of GPIb/IIa, CD63, and P-selectin on platelets after dabigatran treatment (1).
- We further hypothesize that a system biology approach coupled with global proteomics analysis of thrombin activated human platelets treated with dabigatran would reveal the interlinked kinase (ILK) and integrin mediated signaling as major significantly down-regulated pathways by the DTI dabigatran.
- To test our hypothesis, we developed a pharmacoproteomics platform that monitor changes in the protein expression profiles of platelets, purified from healthy human donors and ex-vivo activated in presence or absence of the dabigatran.
- The pharmacoproteins platform employed the nanolC-ESI MS/MS sequencing of tryptic/Glu-C/Lys-C generated peptides from platelets releasates, as well as a Q-Exactive quadrupole orbitrap mass spectrometer coupled with the label free quantitation (LFQ) module provided by PEAKS 8.5/X (Bioinformatics Solutions Inc.).

Background

Figure 1: Joanna von Eyth et al. Frontiers in Pharmacology, February 2013 | Volume 4 | Article 12 | 2

Figure 2: Working model for inhibition of thrombin activated platelets aggregative mediatability dabigatran

Conclusions

This research presents the development, optimization and drug-treatment validation of pharmacoproteomic platforms that monitor changes in the protein expression profiles of platelets, purified from healthy human donors and ex-vivo activated with thrombin in presence or absence of dabigatran (DABII), an FDA approved direct thrombin inhibitor.

The qualitative analysis of the biochemical pathways was accomplished with Ingenuity pathway analysis (IPA, Ingenuity Systems) using the protein ratios extracted from LFQ analyses. In addition cellular pathways monitoring the thrombin activated platelets and GO annotations were accomplished by employing REACTOME and PathTree databases.

The bioinformatics analysis predicted that many proteins involved in ILK and integrin signaling pathways, such as rhoA, rhoGDI, clathrin mediated-endocytosis and ILK and integrin signaling pathways, were at least two-fold statistically significant downregulated (p<0.05) in the dabigatran-treated platelets in comparison to "resting" platelets. Remarkably, the LFQ analysis validated the dabigatran mediated downregulation of many of the already published biomarkers of the interlinked mediated signaling pathways (such as of GPIb/IIa) and FVIIa)

Figure 3: | Label free quantitative proteomics LFQ analysis of whole human platelet samples from three different pharmacoproteomic platforms (A) DABII treated platelet samples vs control untreated platelet samples (B) control untreated platelet samples vs "resting" platelet samples (C) DABII treated platelet samples vs "resting" platelet samples.

Figure 4: | Bioinformatics and IPA analysis of LFQ data from the proteomics profiling of platelets treated with DABII that reveals significant changes in the protein expression levels of platelet proteins involved in cell adhesion (integral), cell movement and aggregation (A: inhibition of platelet aggregation), cytoskeleton rearrangement (B: signaling in response of re-organized platelet kinesins proteins in DABII-treated vs control untreated platelets).

Figure 5: | Working flowchart employed for global proteomics analysis and label free quantitation (LFQ) of changes in the protein expression profiles of healthy human platelets control or treated with direct thrombin inhibitors.

Figure 6: | Protein expression fold changes and LFQ spectral counts for thrombin DABII treated platelet samples (A) and "resting" platelet samples (B) generated using PEAKS X Software.

Figure 7: | Thrombin DABII treated platelet samples vs "resting" platelet samples generated using PEAKS X Software.