**Report from 19th Congress of the European Hematology Association – 12-15th June 2014, Milan, Italy - Magdalena Winiarska**

The annual Congress of the European Hematology Association held this year in Milan is the most important European conference regarding latest advances in hematology. 19th EHA congress gathered more than 9,500 participant not only from Europe but also USA, Japan or Australia. During this meeting I had a great pleasure to present the results of our research project during e-poster session *Gene therapy, immunotherapy and vaccination*. It is worth mentioning that the selection of abstract was peer-reviewed and on a base of the scientific quality abstracts were allocated as follows: 9% Oral Presentation, 47% Poster Presentation, 30% Publication Only and 14% is rejected. The e-poster session which was this year's novelty on EHA and I have to admit it was a really interesting opportunity to present my data during a short 5 minutes long presentation from an e-screen. Moreover, the conference was for me a great chance to listen to participate in many remarkable lectures and to establish future collaboration with other scientists.

Active participation – co-author of two posters presented during poster sessions.

1. Chronic lymphocytic leukemia and related disorders - Biology 1 - Presenting author

2. Gene therapy, cellular immunotherapy and vaccination – Co- author

# SRC inhibitors downregulate CD20 and modulate the activity of the CD20 promoter

P211

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## INTRODUCTION

Anti-CD20 monoclonal antibodies have made a breakthrough in the treatment of non-Hodgkin's lymphoma and chronic lymphocytic leukemia. They trigger indirect effector mechanisms of the immune system, namely complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and immunophagocytosis. Although for many years CD20 has been described as a stable antigen, accumulating evidence indicates that CD20 can be modulated at both transcriptional and posttranscriptional levels. Down-regulation of surface CD20 levels has been linked with tumor resistance to rituximab. Here, we demonstrate that inhibition of Src family kinases (SFK) results in increased resistance of tumor cells to antitumor activity of anti-CD20 mAbs. Our observations strongly imply that CD20 down-regulation relies on transcriptional mechanisms and highlight the role of AKT in SFKs-dependent transcriptional regulation of CD20.

## OBJECTIVES

The aim of this study was to investigate in more detail the molecular basis of Src family tyrosine kinases-dependent regulation of CD20 levels and the influence of Src family kinases inhibitors on antitumor activity of anti-CD20 monoclonal antibodies in models of CD20-positive B-cell malignancies.

## RESULTS

### Fig.1 SFKs inhibitors downregulate surface CD20 levels and affect CDC in Raji cells

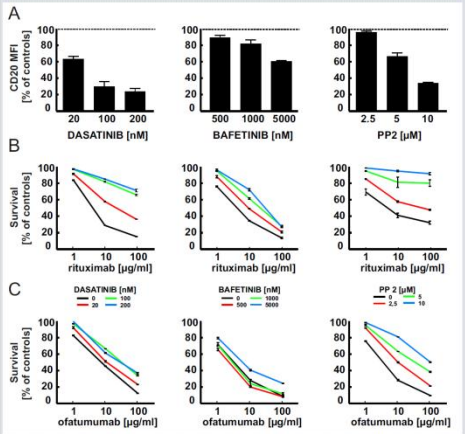


**Fig.1** Raji cells, pretreated for 48 hours with various Src family kinases inhibitors, were incubated with saturating amount of FITC-conjugated anti-CD20 mAb for 30 min at RT in the dark (A), with increasing concentrations of rituximab (B) or increasing concentrations of ofatumumab and 10% human AB serum for 60 min. Binding of antibody was determined with flow cytometry. CDC was measured with PI staining using flow cytometry.

### Fig.2 SFKs inhibitors impair NK cell cytotoxicity in ADCC assay

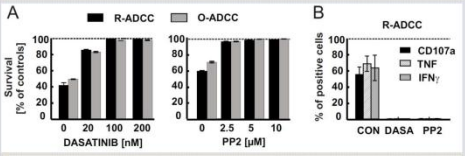


**Fig.2** CFSE-stained Raji cells were co-incubated for 4h with either rituximab or ofatumumab (100 μg/ml) and NK cells at E:T ratio 6:1 in presence of dasatinib or PP2. Raji cell survival was determined with flow cytometry after staining with PI (A). For degranulation assay NK and Raji cells were incubated at E:T ratio 1:1 with rituximab and either dasatinib or PP2 for 4h. NK cells were co-incubated with GolgiStop, anti-CD107a antibody, anti-CD56, anti-CD3 and Fixable Viability Dye. To determine cytokines production cells were permeabilized and stained with anti-IFN-γ or anti-TNF-α antibodies followed by flow cytometry analysis. Results are presented as a percentage of CD107a, TNF-α or IFN-γ positive NK cells (B).

### Fig.3 Dasatinib impairs antitumor activity of rituximab in *in vivo* model

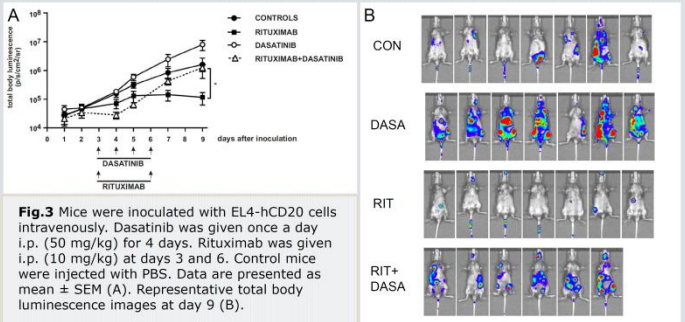


**Fig.3** Mice were inoculated with EL4-hCD20 cells intravenously. Dasatinib was given once a day i.p. (50 mg/kg) for 4 days. Rituximab was given i.p. (10 mg/kg) at days 3 and 6. Control mice were injected with PBS. Data are presented as mean ± SEM (A). Representative total body luminescence images at day 9 (B).

### Fig.4 Dasatinib down-regulates CD20 at protein and mRNA levels.

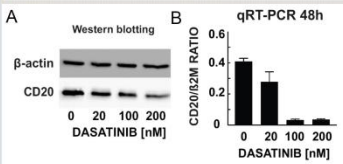


**Fig.4** Raji cells were incubated for 48 hours with increasing concentrations of dasatinib. Total protein lysates were separated in a polyacrylamide gel and analysed for CD20 and actin expression by Western blotting with specific antibodies (A).
cDNA was used for quantitative real-time PCR (qRT-PCR) amplification with SYBR Green Master Mix (B).

### Fig.5 Modulation of CD20 expression by dasatinib requires CD20 promoter

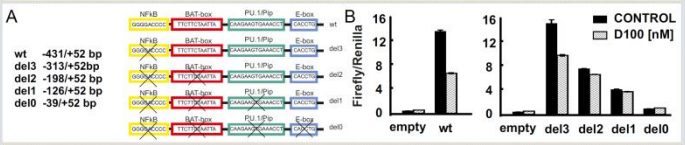


**Fig.5** Scheme of truncated CD20 promoters used for reporter assays (A). Relative luciferase activity was measured in lysates from Raji cells transfected with empty, wild type or truncated pGL4-CD20 promoters incubated with dasatinib for subsequent 24h (B).

### Fig.6 Effects of SFKs inhibitors could be overcome by activation of AKT

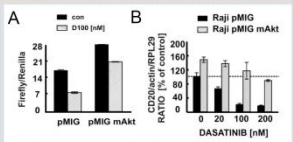


**Fig.6** Relative luciferase activity was measured in protein lysates of Raji cells co-transfected with pGL4-wt CD20 promoter and either pMIG or pMIG-myrAKT and incubated with dasatinib for 48h (A). cDNA from Raji cells stably transduced with either pMIG or pMIG-myrAKT pre-incubated for 24h with dasatinib was used for qRT-PCR amplification of CD20, ACTB and RPL29 products (B).

## CONCLUSIONS

Our studies indicate for the first time that Src family tyrosine kinases are involved in the transcriptional regulation of CD20 levels in lymphoma cells. SFKs inhibitor dasatinib strongly impairs antitumor efficacy of anti-CD20 monoclonal antibodies, both *in vitro* and *in vivo*.

The research was supported by National Science Center 2012/07/B/NZ6/03498 (MW) and Polish Ministry of Science and Higher Education IP2011 060271 (MW) and the European Commission 7th Framework Programme: FP7-REGPOT-2012-CT2012-316254-BASTION.
All authors declared no relevant financial relationship to disclose as well as no off-label drug usage to be included into presented data.

TITLE: HDAC6 inhibition augments the efficacy of anti-CD20 monoclonal antibodies by up-regulating CD20 level in malignant B-cells

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# HDAC6 inhibition augments the efficacy of anti-CD20 monoclonal antibodies by up-regulating CD20 level in malignant B-cells

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## BACKGROUND

CD20, an integrate membrane protein expressed on the surface of normal and malignant B-cells, is widely used as a molecular target for monoclonal antibodies (mAbs) in the therapy of NHL and CLL. Unfortunately, about 50% of patients does not respond to anti-CD20 mAbs. One of the reasons of this resistance is low CD20 level. Accumulating evidence indicates that CD20 can be modulated at several levels, both transcriptional and posttranscriptional and its up-regulation results in increased efficacy of anti-CD20 mAbs. CD20 expression is reported to be regulated epigenetically e.g. by histone deacetylases (HDACs).

## OBJECTIVE

The aim of our project was to check if specific inhibition of HDAC6 can influence CD20 level and improve the efficacy of anti-CD20 mAbs.

### Fig.1. Pan-HDAC inhibitors and specific HDAC6 inhibitors up-regulate CD20 level and improve R-CDC

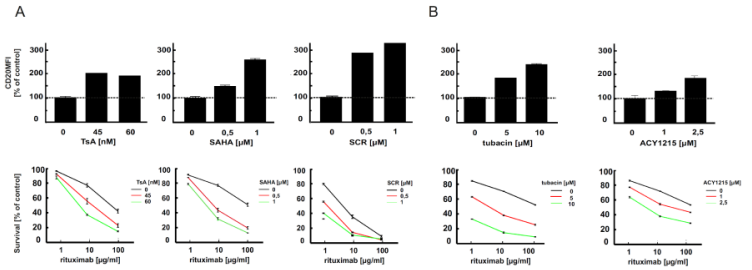


Fig. 1. Raji cells, pretreated for 48 hours with (A) HDAC pan-inhibitors –Trichostatin A (TsA), suberoylanilide hydroxamic acid (SAHA) and Scriptaid (SCR) and (B) specific HDAC6 inhibitors - tubacin and ACY1215 were incubated with FITC-anti-CD20 mAb. Binding of mAb was determined with flow cytometry. Efficacy od rituximab-mediated CDC was assessed with PI staining using flow cytometry after 1h incubation with serial dilutions of rituximab in the presence of 10% human AB serum as a source of complement.

### Fig. 2. Inhibition of HDAC6 up-regulates CD20 level in lymphoma cell lines, EBV transformed B-cells and primary CLL cells

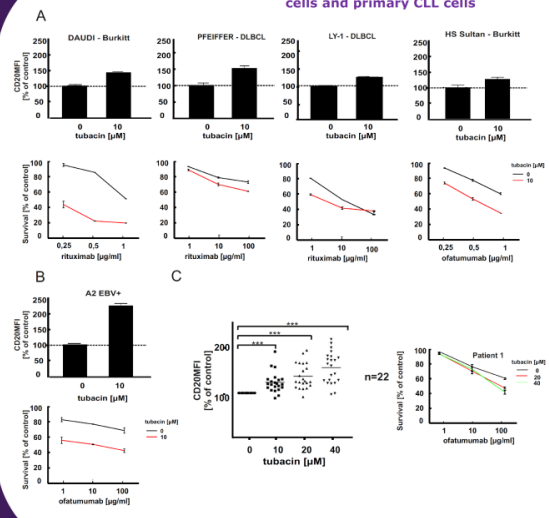


Fig. 2 (A) Lymphoma cell lines – Daudi, Pfeiffer, Ly-1 and HS-Sultan and (B) EBV-transformed B-cells were analysed for surface CD20 by flow cytomtery after staining with FITC-CD20 mAb. CDC with rituximab/ofatumumab (depending on the sensitivity of a cell line) was assessed with flow cytometry.

(C) Primary CLL cells isolated from untreated patients were assayed for surface CD20 using flow cytometry. Results are presented as % of control MFI. The differences in CD20 MFI between control and drug-treated groups were statistically significant (*p<0.0001) as measured using Wilcoxon signed rank test. R-CDC was assessed with flow cytometry as described previously.

## CONCLUSIONS

The results of our study strongly suggest that combining HDACi with anti-CD20 antibodies can be a successful therapeutic modality for patients suffering from B-cell malignancies. The use of isoform-selective inhibitors of HDAC6 may be an effective strategy in enhancing the efficacy of anti-CD20 mAbs. Potentially these compounds would have less adverse effects than HDAC pan-inhibitors. However, their use in the therapy requires further investigation.

The research was supported by Polish Ministry of Science and Higher Education [grants 1M19/3DG4/12 and 2013/09/N/NZ3/01407 (M.B) and IP2011 060271 (M.W).]This work was also supported by a grant from the European Commission FP7-REGPOT 7th Framework Programme: T-2012-CT2012-316254-BASTION. All authors declared no relevant financial relationship to disclose as well as no off-label drug usage to be included into presented data.

### Fig. 3. CD20 up-regulation by HDAC6 inhibitors does not rely on regulation of transcription

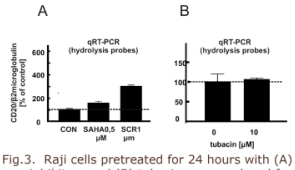


Fig.3. Raji cells pretreated for 24 hours with (A) HDAC pan-inhibitors and (B) tubacin were analyzed for CD20 expression in qRT-PCR with hydrolysis probes.

### Fig.4. HDAC6 silencing augments CD20 and improves R-CDC while HDAC6 overexpression does not affect it

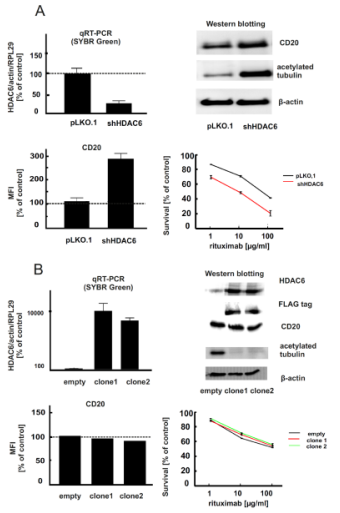


Fig.4. Raji cells were stably transduced with (A) pLKO.1 shHDAC or empty vector and (B) SSFV HDAC6-FLAG or empty plasmid. Level of HDAC6, acetylated-tubulin (a hallmark of HDAC6 inhibition), FLAG-tag, CD20 and actin was assessed with WB. Overexpression/silencing was measured in qRT-PCR. CD20 level and R-CDC were assessed with flow cytometry.

### Fig.5. HDAC6 is implicated in CD20 trafficking

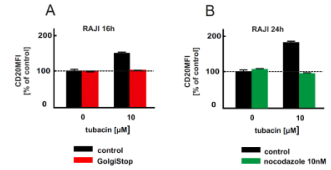


Fig.5. Raji cells were coincubated with (A) protein transport inhibitor GolgiStop for 16h and (B) microtubule destabilizing agent nocodazole 10nM for 24h. The effect of the drugs on the effect of tubacin on CD20 expression was assessed with flow cytometry.