Report on 19th Congress of the European Hematology Association, Milan, Italy, June 12-15, 2014

Jakub Golab

The annual Congress of the European Hematology Association is the most important European conference regarding latest advances in hematology. Over 2300 abstracts have been submitted for inclusion in the program of 19th EHA congress, and only 200 abstracts were selected for a presentation in one of the 40 oral sessions covering all fields of hematology. During the meeting three posters where I was a senior author were presented by members of my team and I participated in the preparation of all of them. The annual EHA Congress is an excellent opportunity to meet cooperating international researchers as well as scientists working in a the field of our studies. It is also a unique opportunity to hear new lectures on the most up-to-date issues in hematooncology.

Poster list:

(1) SK053 – an allosteric protein disulphide isomerase inhibitor induces differentiation of human acute myeloid leukemia cells
(2) HDAC6 inhibition augments the efficacy of anti-CD20 monoclonal antibodies by up-regulating CD20 level in malignant B-cells
(3) Src inhibitors downregulate CD20 and modulate the activity of the CD20 promoter

SK053 – an allosteric protein disulphide isomerase inhibitor induces differentiation of human acute myeloid leukemia cells

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Background: Introduction of differentiation-inducing agents including all-trans retinoic acid (ATRA) and arsenic trioxide to the treatment of acute promyelocytic leukemia (APL) was a remarkable therapeutic breakthrough resulting in cure rates exceeding 80%. However, there is no such significant progress in the treatment of other acute myeloid leukemia (AML) types. Thus search for new agents exerting anti-leukemic effects by targeting novel and unique cellular mechanisms is of utmost clinical importance. Numerous human proteins involved in tumor formation contain allosteric disulfide bonds that are cleaved by oxidoreductases or by thiol-disulfide exchange. Such disulfide modifications participate in post-translational protein control and affect protein function. Targeting of allosteric disulfide bonds is a novel promising strategy in cancer therapy (*Hogg P, Nat Rev Cancer 2013*). We have recently developed SK053, a small molecule inhibitor of thioredoxin/thioredoxin reductase system, that showed anti-tumor effects both *in vitro* and in murine tumor models (*Klossowski S, Muchowicz A et al., J Med Chem 2012*). Our ongoing studies revealed that SK053 is not a target-specific, but mechanism-selective inhibitor of enzymes involved in allosteric disulfide bonds formation such as protein disulfide isomerase (PDI).

The aim of the studies was to determine whether targeting the formation of allosteric disulfide bonds with SK053 can induce antitumor effects in acute myeloid leukemia.

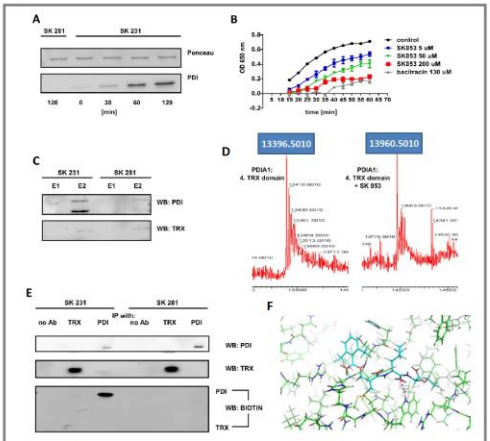

Fig.1. SK053 binds to PDI and inhibits its enzymatic activity.
(A) binding of biotinylated active SK053 analog - SK231 and biotinylated inactive compound - SK28 to recombinant PDI protein; **(B)** SK053 inhibits the enzymatic activity of PDI in a turbidimetric assay of insulin disulfide reduction, bacitracin served as a positive control; **(C)** precipitation of biotinylated proteins using neutravidin-coated beads from the lysates of HL60 cells preincubated for 4 h with 100 μM SK231; elution with 5 mM biotin (E1) and 5 mM biotin + 5 M urea (E2). The presence of PDI and TRX was detected with Western blotting; **(D)** 4th thioredoxin domain of recombinant PDI was incubated with 10× molar excess of SK053 and analyzed by mass spectrometry; **(E)** immunoprecipitation of indicated proteins from the lysates of HL60 cells preincubated for 4 h with 100 μM SK231, presence of PDI and TRX, and biotin was detected with Western blotting; **(F)** covalent docking of SK053 (cyan) to the Cys400 in the 4th thioredoxin domain of human PDI, key role of R461, H399 i W396, F304, F428; GOLD docking software, analysis done by Michal Lazniewski, PhD, from the Interdisciplinary Centre for Mathematical and Computational Modelling, University of Warsaw, Poland.

In summary, SK053 targets PDI and thioredoxin/thioredoxin reductase system, has significant anti-leukemic activity and induces differentiation of various types of human AML cells. Thus, targeting of enzymes involved in allosteric disulfide bonds formation with small molecule inhibitors presents a novel and promising therapeutic strategy in acute myeloid leukemia.

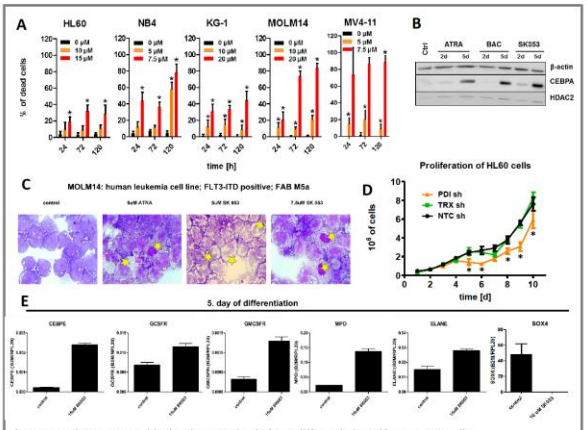

Fig. 2. SK053 is a potent anti-leukemic agent that induces differentiation of human AML cells.
(A) Cytostatic/cytotoxic activity of SK053 evaluated with trypan blue exclusion assay, the graph presents mean percantage of dead cells ± SD, n = 6; *p<0.05 one-way ANOVA with Dunnett's post hoc test; **(B)** CEBPA expression in nuclear lysates of HL60 cells incubated with 1 μM ATRA, 2 mM bacitracin or 10 μM SK053; **(C)** cytospin and May-Grunwald-Giemsa staining of MOLM14 cells incubated for 5 days with indicted concentrations of SK053 or 5 μM ATRA. Asterisks indicate cells with morphological signs of differentiation; **(D)** proliferation of HL60 cells transduced with non-targeting (NTC), TXN and PDI targeting shRNA lentiviral particles (Sigma-Aldrich), cells counted daily in trypan blue, *p<0.05 vs NTC, one-way ANOVA; **(E)** HL60 cells were incubated for five days with 10 μM SK053, qPCR results presented as mean target-to-reference ratio ± SD.

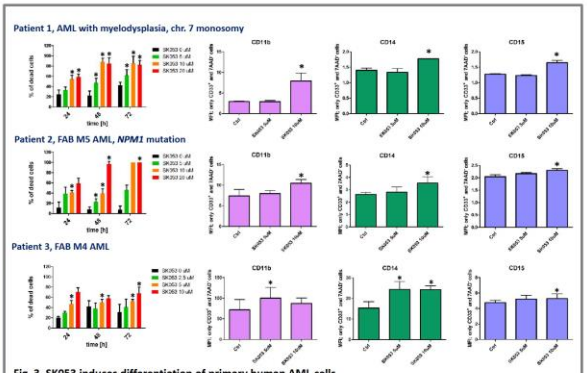

Fig. 3. SK053 induces differentiation of primary human AML cells.
(*Left panel*): Cytostatic/cytotoxic activity of SK053 evaluated with trypan blue exclusion assay, the graph presents mean percantage of dead cells ± SD, n = 6; *p<0.05 one-way ANOVA with Dunnett's post hoc test; (*Right panel*): Expression of differentiation markers in flow cytometry after 3 days of incubation of AML cells with SK053; *p<0.05 one-way ANOVA with Dunnett's post hoc test.

All the authors declared no relevant financial relationship to disclose as well as no off-label drug usage to be included into presented data.

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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

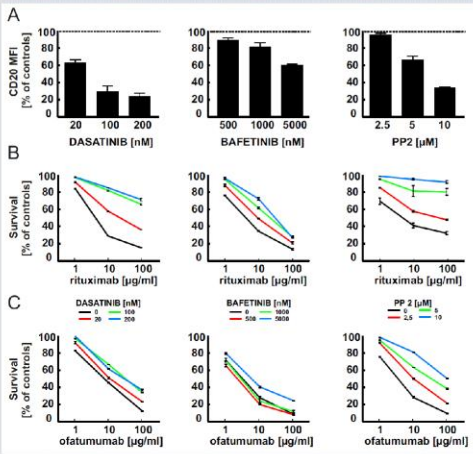

Fig.1 Raji cells, pretreated for 48 hours with various Src family kinases inhibitors, were incubated with saturating amount of FITC-conjugated anti-CD20 mAb for 30 min at RT in the dark (A), with increasing concentrations of rituximab (B) or increasing concentrations of ofatumumab and 10% human AB serum 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

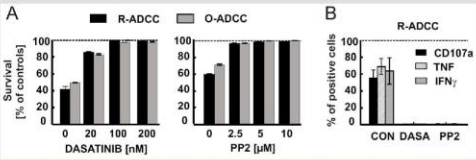

Fig.2 CFSE-stained Raji cells were co-incubated for 4h with either rituximab or ofatumumab (100 μg/ml) and NK cells at E:T ratio 6:1 in presence of dasatinib or PP2. Raji cell survival was determined with flow cytometry after staining with PI (A). For degranulation assay NK and Raji cells were incubated 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

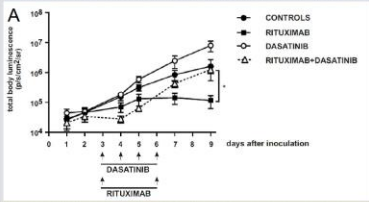

Fig.3 Mice were inoculated with EL4-hCD20 cells intravenously. Dasatinib was given once a day i.p. (50 mg/kg) for 4 days. Rituximab was given i.p. (10 mg/kg) at days 3 and 6. Control mice were injected with PBS. Data are presented as mean ± SEM (A). Representative total body luminescence images at day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

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

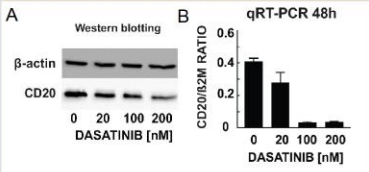

Fig.4 Raji cells were incubated for 48 hours with increasing concentrations of dasatinib. Total protein lysates were separated in a polyacrylamide gel and analysed for CD20 and actin expression by Western blotting with specific antibodies (A).
cDNA was used for quantitative real-time PCR (qRT-PCR) amplification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

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

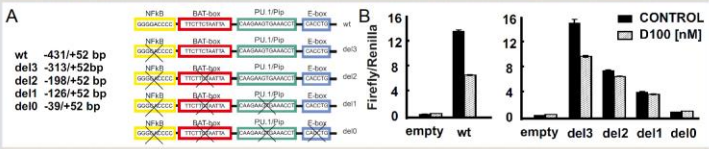

Fig.5 Scheme of truncated CD20 promoters used for reporter assays (A). Relative luciferase activity was measured in lysates from Raji cells transfected with empty, wild type or truncated pGL4-CD20 promoters incubated with dasatinib for subsequent 24h (B).

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

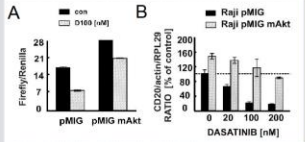

Fig.6 Relative luciferase activity was measured in protein lysates of Raji cells co-transfected with pGL4-wt CD20 promoter and either pMIG or pMIG-myrAKT and incubated with dasatinib for 48h (A). cDNA from Raji cells stably transduced with either pMIG or pMIG-myrAKT pre-incubated for 24h with dasatinib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

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

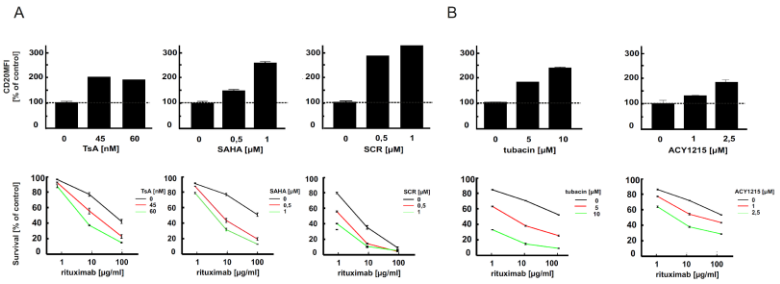

Fig. 1. Raji cells, pretreated for 48 hours with (A) HDAC pan-inhibitors –Trichostatin A (TsA), suberoylanilide hydroxamic acid (SAHA) and Scriptaid (SCR) and (B) specific HDAC6 inhibitors - tubacin and ACY1215 were incubated with FITC-anti-CD20 mAb. Binding of mAb was determined with flow cytometry. 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

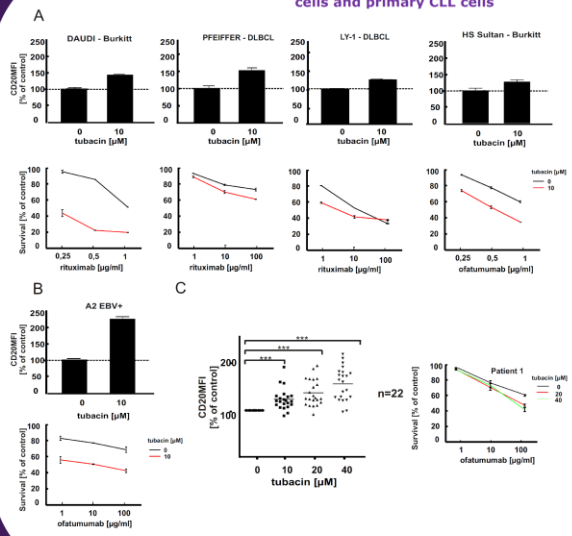

Fig. 2 (A) Lymphoma cell lines – Daudi, Pfeiffer, Ly-1 and HS-Sultan and (B) EBV-transformed B-cells were analysed for surface CD20 by flow cytomtery after staining with FITC-CD20 mAb. CDC with rituximab/ofatumumab (depending on the sensitivity of a cell line) was assessed with flow cytometry.

(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

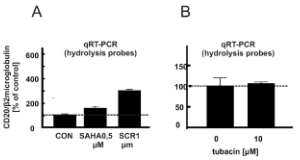

Fig.3. Raji cells pretreated for 24 hours with (A) HDAC pan-inhibitors and (B) tubacin were analyzed for CD20 expression in qRT-PCR with hydrolysis probes.

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

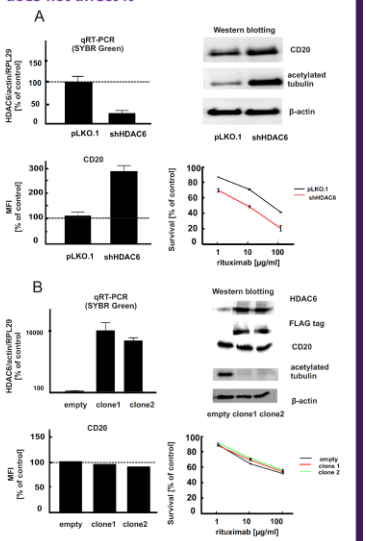

Fig.4. Raji cells were stably transduced with (A) pLKO.1 shHDAC or empty vector and (B) SSFV HDAC6-FLAG or empty plasmid. Level of HDAC6, acetylated-tubulin (a hallmark of HDAC6 inhibition), FLAG-tag, CD20 and actin was assessed with WB. Overexpression/silencing was measured in qRT-PCR. CD20 level and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

Fig.5. HDAC6 is implicated in CD20 trafficking

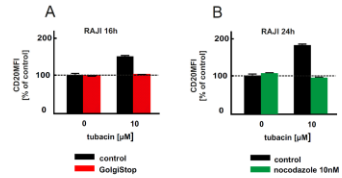

Fig.5. Raji cells were coincubated with (A) protein transport inhibitor GolgiStop for 16h and (B) microtubule destabilizing agent nocodazole 10nM for 24h. The effect of the drugs on the effect of tubacin on CD20 expression was assessed with flow cytometry.