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Differential Regulation Patterns of Anti-CD20 Antibodies Obinutuzumab and Rituximab in Mantle Cell Lymphoma

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Introduction

Mantle cell lymphoma (MCL) is an aggressive B-cell Non-Hodgkin lymphoma characterized by a poor long-term prognosis.(Dreyling and Hiddemann 2009) The most important step in improving the outcome in MCL has been the introduction of the type I anti-CD20 monoclonal antibody (mAb) rituximab. In this study we focus on the comparison of type I mAb rituximab and novel humanized and glycoengineered type II obinutuzumab. In order to reveal common and distinct downstream pathways, we conducted an *in-vitro* study in isolated MCL cell lines after rituximab or obinutuzumab mono- and combination-treatment.

Materials and Methods

MCL cell lines Granta-519, Jeko-1 Rec-1 and Z-138 were provided by the DSMZ (Braunschweig, Germany) or the respective primary investigator. Experiments were performed in triplicates. MCL cells were treated with obinutuzumab and / or rituximab at 10 µg/ml. Cell viability was analysed by “ViCell Cell Viability Analyzer” tests at 0h, 24h, 48h and 72h. Fractional product was calculated: synergism > 0,1; antagonism < -0,1. Cell preparation for Affymetrix analysis was performed according to the manufacturer’s protocol. The cells were treated for 4h with 10 µg/ml obinutuzumab, rituximab, human anti-IgG antibody (isotype control) and PBS (buffer control, pH 7.2, 7µl). Genechip HU U133 Plus 2.0 was used for cRNA microarray analysis. All samples fulfilled the filter criteria: fold change > 1, mean > 100, call > 0.5 and t-test p-value < 0.05. Networks were generated through the use of IPA (Ingenuity® Systems).

Results and discussion

In-vitro effects of obinutuzumab and rituximab

Obinutuzumab induced a higher reduction in cell viability in each MCL cell line than rituximab. (Figure 1) Combination experiments suggested the competitive binding of the two antibodies due to overlapping epitopes on the target cells and resulted in a lower cytotoxicity than obinutuzumab alone, according to fractional product calculations (Granta-519: -0.56; Jeko-1: -0.14; Rec-1: -0.44; Z-138: -0.18). Due to the high rate of cell kill in Granta-519, micro-array data collection was not possible.

Commonly deregulated genes after both mono-treatments

19 of the 21 commonly deregulated genes were also deregulated after combination therapy. Cytokines CCL3 and CCL4 are upregulated in both mono-experiments. Cittera et al. identified the upregulation of cytokines CCL3 and CCL4 after rituximab administration, presumably involved in the eradication of lymphoma cells.(Cittera*, et al* 2007) Francke et al. were able to show that treatment with anti-CD20 and B-cell receptor (BCR) mAbs resulted in a similar deregulation of the transcriptome in multiple lymphoma cell lines.(Franke*, et al* 2011)

Both mAb treatments share an upregulation of cancer suppressor gene *EGR-1* that also belongs to the BCR signalling pathway, known to down-regulate *Survivin*, an anti-apoptotic molecule, and to induce caspase signalling resulting in apoptosis induction.(Chen*, et al* 2010) *Survivin* was silenced by siRNA in MCL cell line Jeko-1, followed by a reduction in proliferation.

Both treatments upregulated *DUSP2*, a gene that belongs to the ERK/MAPK pathway that plays a crucial role in growth suppression.

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Obinutuzumab-only treatment uniquely deregulated a subset of 21 genes. It induced an upregulation of transcription factor *AP-1*, that is comprised of *JUN* and *FOS* family members, forming a heterodimer. *AP-1* is involved in apoptosis induction, in survival and proliferation of cells.(Shaulian and Karin 2001) Low *JUN*-expression in Multiple Myeloma (MM) was associated with early disease-related deaths.(Chen*, et al* 2010) A down-regulation of *EGR-1* was found to be associated with disease progression in MM patients. *EGR-1* is a direct downstream target gene of *JUN*.(Chen*, et al* 2010) An up-regulated EGR-1 gene induces a down-regulation of *Survivin*.(Wagner*, et al* 2008) In consequence, down-regulation of *Survivin* is followed by an activation of caspase-induced apoptosis.(Chen*, et al* 2010)

In addition, Chen et al. were able to show that *JUN* and *EGR-1* are key role players in the bortezomib induced apoptosis downstream pathway in MM.(Chen*, et al* 2010) Upregulated *NFKBIE* encodes for a protein known to bind to NF-κB components targeting the complex for degradation. As a result, the NF-κB complex is prevented from deregulating genes in the nucleus and promoting cell proliferation. The encoded protein *I□B* is deactivated by phosphorylation mediated by IκB-kinases. Apart from NF-□B-signalling, together with *NFATC1, NFKBIE* is involved in PI3K-signalling. (Table 1b)

Uniquely deregulated genes after rituximab mono-treatment

Rituximab mono-treatment resulted in 29 uniquely altered genes. Rituximab's intermediate *in-vitro* effect might be in part due to further induction of the already overexpressed WNT/ß-catenin canonical pathway, a key pathway in MCL pathogenesis.(Rizzatti*, et al* 2005) Upregulation of *WNT* and *SOX11* might indicate a cellular response leading to less pronounced results in apoptosis induction.

Downregulated *BCL2A* belongs to a group of cell death regulators, involved in the NF-κB pathway facilitating cell-survival in healthy and cancer cells.(Vogler 2012). Vogler argues that the development of small molecule inhibitors for this target gene might contribute to anti-tumour therapy.(Vogler 2012) (Table 1c)

Deregulated genes by combination treatment

The combination experiments disclosed 37 up- and 6 downregulated genes. 16 genes were unique to both rituximab mono- and combination treatments, while obinutuzumab and combination exposure shared only two uniquely deregulated genes, indicating a rituximab-like expression pattern in combination experiments, similar to the *in-vitro* findings. Both mAbs have approximately the same affinity (obinutuzumab: 4.0 nM vs. rituximab: 4.5 nM K_D value) to the human CD20. The *in-vitro* effect is related to the overlapping epitopes, although it may be argued that on a particular cell CD20 molecules can be occupied by rituximab and obinutuzumab simultaneously.(Mossner*, et al* 2010) *Niederfellner et al*. hypothesized that the excess binding sites for type I mAbs are either due to an abundance of various CD20 molecules that are targeted by either mAb type or due to steric binding orientations and elbow-hinge angle conformations.(Niederfellner*, et al* 2011) Furthermore, the CD20 epitope displays twice as many binding sites for type I mAbs as for type II. (Mossner*, et al* 2010, Niederfellner*, et al* 2011)

In conclusion, new combination experiments are needed to reveal potential additive effects in MCL therapy. PI3K-inhibitors such as idelalisib, proteasome-inhibitors such as bortezomib, anti-BCR mAbs, siRNA of *Survivin* or IKK-inhibitors are promising combination partners for obinutuzumab.

These findings indicate that a combination with obinutuzumab may be more promising than rituximab in MCL patients. However, patients recently pre-treated with rituximab should only be exposed to obinutuzumab after rituximab levels have decreased. The understanding of the deregulation patterns presented in our work might guide more rational approaches for new combination experiments in MCL.

Acknowledgements and "Conflict of Interest" statement

DH designed the research study, performed the research, analysed the data and wrote the paper. MW acted in a supervisory role in the laboratory and assisted in analysing the data. GH helped to conceptualize the structure of the study. YZ contributed to the execution of the research. VJ also helped to analyse the data. As chief of the department WH helped conceptualize the study and provided essential reagents or tools. MD acted in an advisory role, designed the research study and contributed essential reagents or tools. The "Lymphoma Research Foundation" and the "European Mantle Cell Lymphoma Network" supported this work.

DH declares to have a patent pending on obinutuzumab combination treatment. MW, GH, YZ, VJ declare no potential conflict of interest. WH declares speaker's honoraria, support of investigator-initiated trials from Roche. He is on the scientific advisory board of Roche. MD declares to receive speaker's honoraria, support of investigator initiated trials from Roche. He declares to be on the scientific advisory board of Roche. He declares to have a patent pending on obinutuzumab combination treatment.

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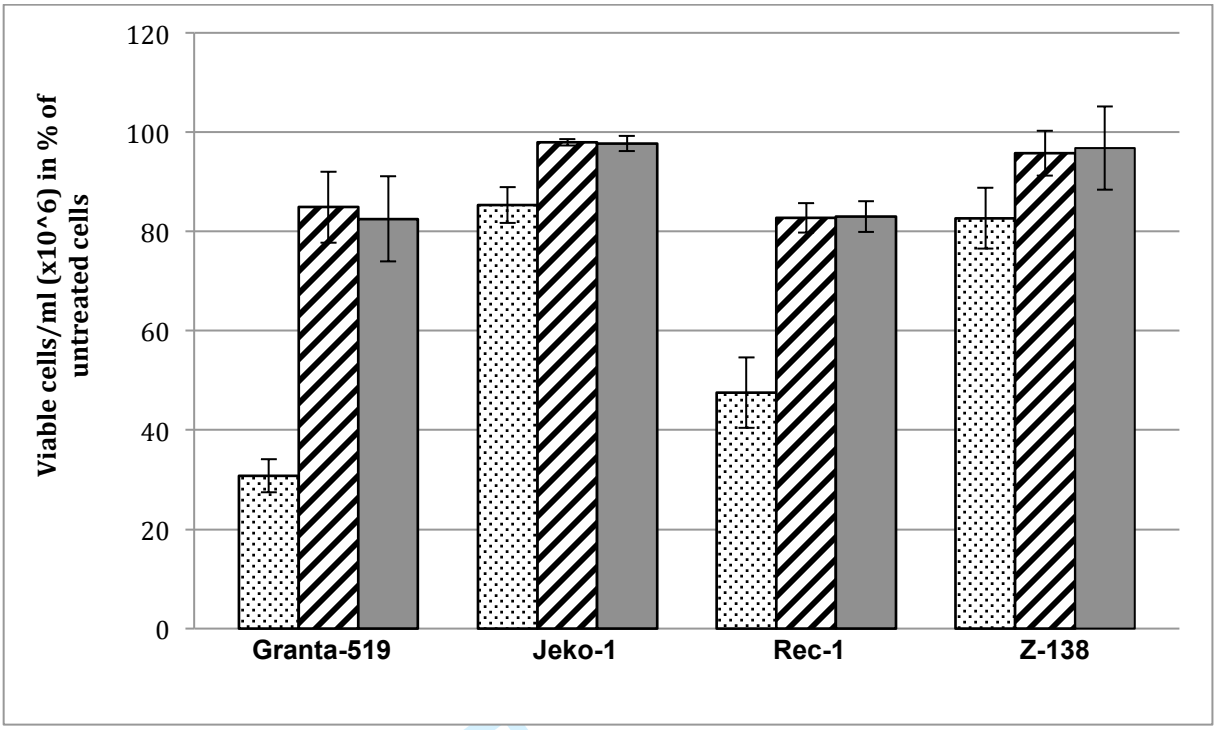

Figure 1: In-vitro reduction of cell viability of MCL cell lines Granta-519, Jeko-1, Rec-1 und Z-138 after obinutuzumab (dots), rituximab (diagonal lines) and combination therapy (dark grey) at equimolar concentrations (10 µg/ml) at 72 hours after treatment. The indicator of error displays standard deviation by percentage.

Table 1a

Commonly deregulated genes after rituximab or obinutuzumab mono-treatment

Symbol	Entrez Gene Name	Location	Type(s)	up- / down-regulated
CCL3	chemokine (C-C motif) ligand 3	Extracellular Space	cytokine	↗
CCL4	chemokine (C-C motif) ligand 4			↗
CSF1	colony stimulating factor 1 (macrophage)			↗
MAP2	microtubule-associated protein 2	Cytoplasm	other	↗
NR4A3	nuclear receptor subfamily 4, group A, member 3	Nucleus	ligand-dependent nuclear receptor	↗
HIST1H1D	histone cluster 1, H1d		other	↗
HIST1H2AJ	histone cluster 1, H2aj			↗
HIST1H3A (includes others)	histone cluster 1, H3a			↗
RGS2	regulator of G-protein signaling 2, 24kDa			↗
MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)			↘
DUSP2	dual specificity phosphatase 2		phosphatase	↗
DUSP5	dual specificity phosphatase 5			↗
EGR1	early growth response 1		transcription regulator	↗
EGR2	early growth response 2			↗
EGR3	early growth response 3			↗
TFEC	transcription factor EC			↗
AMIGO2	adhesion molecule with Ig-like domain 2	Plasma Membrane	other	↗
RGS1	regulator of G-protein signaling 1			↗
SPRED1	sprouty-related, EVH1 domain containing 1			↗
SPRY2	sprouty homolog 2 (Drosophila)			↗
SLC34A3	solute carrier family 34 (sodium phosphate), member 3		transporter	↘

Table 1b

Uniquely deregulated genes after obinutuzumab mono-treatment

Symbol	Entrez Gene Name	Location	Type(s)	up- / down-regulated
IER2	immediate early response 2	Cytoplasm	other	↗
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2		enzyme	↘
SOCS3	suppressor of cytokine signaling 3		phosphatase	↗
C16orf54	chromosome 16 open reading frame 54	Extracellular Space	other	↗
DKK2	dickkopf 2 homolog (Xenopus laevis)			↗
PDGFA	platelet-derived growth factor alpha polypeptide		growth factor	↗
FOS	FBJ murine osteosarcoma viral oncogene homolog	Nucleus	transcription regulator	↗
IKZF2	IKAROS family zinc finger 2 (Helios)			↘
NAB2	NGFI-A binding protein 2 (EGR1 binding protein 2)			↗
NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1			↗
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon			↗
PPIL4	peptidylprolyl isomerase (cyclophilin)-like 4		enzyme	↘
CCNG2	cyclin G2		other	↘
ZBTB24	zinc finger and BTB domain containing 24			↗
ANXA1	annexin A1	Plasma Membrane	other	↗
CD69	CD69 molecule	Plasma Membrane	transmembrane receptor	↗
ICAM1	intercellular adhesion molecule 1			↗
IL21R	interleukin 21 receptor			↗
MIR155HG	MIR155 host gene (non-protein coding)	unknown	other	↗
TMEM107	transmembrane protein 107	unknown	other	↗
FAM65B	family with sequence similarity 65, member B	unknown	other	↘

Table 1c

Uniquely deregulated genes after rituximab mono-treatment

Symbol	Entrez Gene Name	Location	Type(s)	up- / down-regulated
BCL2A1	BCL2-related protein A1	Cytoplasm	other	↘
DCAF12	DDB1 and CUL4 associated factor 12			↗
HSPA1A/HSPA1B	heat shock 70kDa protein 1A			↗
LPL	lipoprotein lipase			↗
RHEBL1	Ras homolog enriched in brain like 1		enzyme	↗
TRIM2	tripartite motif containing 2			↘
PPP2R4	protein phosphatase 2A activator, regulatory subunit 4		phosphatase	↘
CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin-like)	Extracellular Space	kinase	↗
SPRED2	sprouty-related, EVH1 domain containing 2		cytokine	↗
COL4A2	collagen, type IV, alpha 2		other	↘
WNT3	wingless-type MMTV integration site family, member 3			↗
IFIH1	interferon induced with helicase C domain 1	Nucleus	enzyme	↘
ESRRG	estrogen-related receptor gamma		ligand-dependent nuclear receptor	↗
NFKBID	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta		transcription regulator	↘
RELB	v-rel reticuloendotheliosis viral oncogene homolog B			↘
RFX3	regulatory factor X, 3 (influences HLA class II expression)			↗
SOX11	SRY (sex determining region Y)-box 11			↗
TP63	tumor protein p63			↘
CHL1	cell adhesion molecule with homology to L1CAM (close homolog of L1)	Plasma Membrane	other	↗
FAIM3	Fas apoptotic inhibitory molecule 3			↗
LILRA4	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 4			↗
LY9	lymphocyte antigen 9			↗
NINJ1	ninjurin 1			↘
OCA2	oculocutaneous albinism II		transporter	↗
SLC38A1	solute carrier family 38, member 1			↗
HIST1H2AG (includes others)	--	unknown	other	↗
MBNL2	muscleblind-like splicing regulator 2			↗
OTUD1	OTU domain containing 1			↗
UBALD2	UBA-like domain containing 2			↗

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