AKT Modulates Sensitivity of AML Cells to Gemtuzumab Ozogamicin

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Acute myeloid leukemia (AML) is associated with a high rate of resistance to current chemotherapies, necessitating the development of novel therapeutics to treat the disease more effectively. Gemtuzumab ozogamicin (GO) is a conjugate between a calicheamicin derivative, a chemotherapeutic drug that induces DNA damage, and a monoclonal antibody that recognizes CD33 on leukemic blast cells. Clinical studies show that only a subset of AML patients respond to treatment with GO, while the large majority do not. A collaborative study between Dr. Roland Walter's laboratory in the Clinical Research Division and the company Nodality in San Francisco, California, used single-cell network profiling (SCNP) to identify pathways that are activated in GO resistant cells, providing a molecular basis for drug failure, while also providing a prognostic tool to identify patients who will or will not respond to treatment.

GO was originally developed in a collaboration between the laboratory of Dr. Irwin Bernstein (Clinical Research Division) and industry based on in vitro findings suggesting that, in some AML patients, the leukemia derives from more mature CD33-positive precursor cells. The CD33 antibody component of GO targets the leukemic cells, delivering the drug to them specifically. In vitro, GO sensitivity varies over 100,000-fold among primary AML patient cell samples. Dr. Roland Walter in the Clinical Research Division and other researchers previously examined the mechanisms of drug efficacy in correlative and in vitro studies (Walter *et al*., 2012). These studies have shown that GO toxicity depends on the amount of intracellular drug, which is affected by CD33 expression on cells, toxin release and activation, as well as extrusion from the cell by drug efflux pumps (see figure). Once inside the cell, toxicity of calicheamicin is modulated both by the ability of the cell to repair DNA damage, as well as the activity of pro- and anti-apoptotic pathways downstream of DNA damage response. The impact of these pathways on GO-mediated cell death or resistance has not been examined in detail.

In this study, six adult and six pediatric AML patient cells were analyzed by SCNP. Using flow cytometry, the SCNP assay analyzes signaling pathways in individual cells by looking at the activation state of signaling proteins after biological stimulation (Irish *et al*., 2006). Antibodies conjugated to different fluorophores bind to the proteins of interest, and can specifically distinguish protein phosphorylation that indicates activation. Flow cytometric analysis using multiple parameters

can be carried out on a single cell. Subsets of cell populations can be analyzed by selecting for expression of cell surface markers.

Using SNCP, five AML patient cells were classified as sensitive to GO, and seven were resistant using a cutoff of 15% apoptosis at 24 hours. In all twelve AML samples GO induced DNA damage, as detected by phosphorylation of histone H2AX, but did not induce cell death in all. Importantly, the apoptotic machinery was functional in the resistant cells, as another chemotherapeutic agent, staurosporine, was able to induce cell death. CD33 expression did not correlate with cytotoxicity in these samples at 24 hours, indicating other pathways of resistance were at work in these samples. However, DNA damage strongly correlated with CD33 expression levels ($r = 0.831$ [0.492–0.951], $P = 0.0008$), as was found previously. Furthermore, treatment of cells with a drug efflux inhibitor, PSC833, increased DNA damage in pediatric AML patient samples by increasing the intracellular concentration of drug. However, while DNA damage is necessary for GO toxicity, it was not sufficient to induce cell death in two of the AML patient samples.

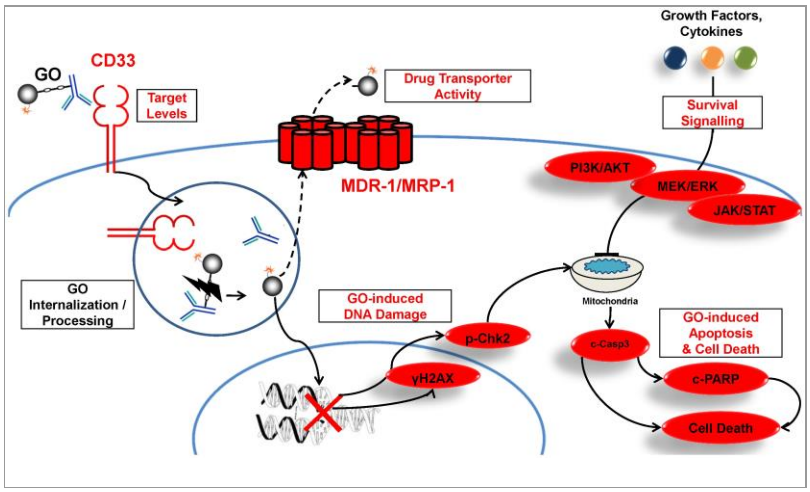
To examine differences in cell proliferation and survival signaling pathways in AML cells, PI3K/AKT, MEK/ERK, and JAK/STAT pathways were examined by looking at the phosphorylation of the respective cell signaling proteins after growth factor treatment. GO sensitive and resistant AML cell lines were initially used to analyze pathways with specific modulators which are important for hematopoietic cell proliferation and survival, in this case the cytokines stem cell factor (SCF) and FLT3 ligand. SCF-induced PI3K pathway activity was increased in all three GO resistant samples relative to GO sensitive samples, as determined by phospho-AKT levels (mean log2-fold increase of 3.33±0.58 *vs*. 0.98±0.69, $P = 0.060$) and phospho-S6 levels (mean log2-fold increase of 1.62±0.18 *vs.* 0.38±0.42, $P = 0.054$). Both proteins were also increased significantly for FLT3 ligand stimulation of pediatric patient cells. Furthermore, the AKT inhibitor MK-2206 sensitized otherwise resistant AML cells lines TF-1 and KG-1 to GO treatment, with and without the drug efflux inhibitor. Taken together, these data suggest the AKT pathway is a rate-limiting step to calicheamicin toxicity.

Future studies on larger numbers of primary AML specimens are required to determine which pathways are relevant in adult AML patients. If validated in clinical samples with known outcomes, SCNP could be used to personalize therapy to patients, by selecting the most appropriate drug combinations. According to Dr. Walter, “this knowledge may turn into improved therapeutic strategies using GO or, by extrapolation, other antibodies that are loaded with a calicheamicin toxin.”

Rosen D.B., Harrington K.H., Cordeiro J.A., Leung L.Y., Putta S., Lacayo N., Laszlo G.S., Gudgeon C.J., Hogge D.E., Hawtin R.E., Cesano A., and Walter R.B. 2013. AKT signaling as a novel factor associated with in vitro resistance of human AML to gemtuzumab ozogamicin. *PLoS One* 8:e53518

Also see: Walter R.B., Appelbaum F.R., Estey E.H., and Bernstein I.D. 2012. Acute myeloid leukemia stem cells and CD33-targeted immunotherapy. *Blood* 119:6198-208.

Irish J.M., Kotecha N., Nolan G.P. 2006. Mapping normal and cancer cell signaling networks: towards single-cell proteomics.*Nature Reviews Cancer* 6:146-55.



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The proposed mechanism of action of GO-induced cell death in CD33-postivive AML cells. GO binds to CD33 positive cells, calicheamicin is activated and released, and DNA damage is induced. The mechanism of GO resistance was determined by SCNP analysis, measuring CD33 expression levels; activity of drug efflux pumps; extent of DNA damage (by H2AX phosphorylation); activation of cell survival signaling pathways (MEK/ERK, PI3K/AKT, and JAK/STAT pathway); and induction of apoptosis and cell death (cleaved PARP and cell membrane integrity).