Research on HDACi synergistic apoptosis induction and cell cycle arrest in B-cell lymphoma cell lines by Department Chair Eric Hsi, MD (l) and Clinical Pathology research fellow Juraj Bodo, PhD, has important clinical implications. Read about their findings, p. 2

Also In This Issue
HEA Assay: Red Cell Blood Group Antigen Typing by DNA Microarray p 5

Feature Story | HDAC Inhibitors Potentiate Enzastaurin’s Apoptotic Effect in Lymphoma p 2
Cover Story

HDAC Inhibitors Potentiate Enzastaurin’s Apoptotic Effect in Lymphoma

By Juraj Bodo, PhD and Eric D. Hsi, MD

Background

The PKC family of isoenzymes is involved in key cellular processes that include cell proliferation, apoptosis, and differentiation through the PI3K/Akt pathway. The role of PKC enzymes in tumorigenesis suggests that combining PKC inhibitors with conventional treatment or new therapeutic agents may increase the efficacy of anti-cancer treatment (1). Evidence from preclinical studies further supports this as a promising clinical approach.

Enzastaurin is a potent and selective oral inhibitor of several PKC isoforms with anti-proliferative activity (2). Studies have demonstrated that this inhibitor is well tolerated, both as a monotherapy and in combination with chemotherapy. Prolonged courses of enzastaurin augment chemotherapy or radiation-induced tumor inhibition of glioma, breast, and small cell lung cancer cell xenografts (3). Based on promising preclinical data such as these, enzastaurin is under evaluation in late-stage clinical trials for the treatment of diffuse large B-cell lymphoma (DLBCL) and glioblastoma.

Histone deacetylase inhibitors (HDACi) are a structurally diverse group of compounds that inhibit HDACs (one of the major enzyme families involved in epigenetic control). These inhibitors represent an emergent class of therapeutic agents that induce tumor cell cycle arrest and apoptosis in various hematologic malignancies. One of them, vorinostat (SAHA) is approved in the United States for the treatment of cutaneous T-cell lymphoma (CTCL) as a single agent. Recently it has been shown that broad spectrum HDACi, which induce tumor cell cycle arrest and apoptosis in various hematologic malignancies, significantly increases expression of PKCbeta (4). Active PKCbeta led to increased survival and proliferation signals, such as phospho-GSK3beta (S9), which may be one of the mechanisms antagonizing the apoptotic effect of HDACi.

SAHA/enzastaurin combination induces caspases-dependent apoptosis

We investigated the cytotoxicity and mechanisms of cell death of the combination of enzastaurin and low concentrations of HDACi in B-cell and T-cell lymphoma cell lines and primary lymphoma/leukemia cells.

Combined enzastaurin/SAHA treatment synergistically induced apoptosis in treated germinal center B-cell (GCB)-like lymphoma cell lines, activated B-cell (ABC)-like lymphoma cell lines, T-cell lymphoma cell lines, and primary lymphoma/leukemia samples. Similarly, a combined treatment of B-cell-like lymphoma cells with enzastaurin and two different HDAC inhibitors (valproic acid and CHAHA) synergistically induced apoptosis, suggesting the synergy can be generalized to other HDAC inhibitors.

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After 48 hours, a significant increase in the percentage of apoptotic cells was detected in the SU-DHL-6 and OCI-LY-19 GCB-like lymphoma cell lines with the combined SAHA/enzastaurin treatment (Figure 1). In the SU-DHL-6 cell line treated with 2.5 µM enzastaurin, we detected 5.1% necrotic and 13.5% apoptotic cells. However, if these cells were treated simultaneously with 0.5 µM SAHA, we detected 5.3% necrotic and 34.0% apoptotic cells. In OCI-LY-19 cells the difference between enzastaurin single treatment and combined treatment was 9.5% for the necrotic population and 24.7% for the apoptotic population. Calculation of the combination index for these combinations showed a CI < 1.0, confirming the synergy between the two agents (not shown).

Apoptosis is associated with caspase activation and G2/M cell cycle arrest

To understand the apoptotic mechanisms at play, we performed Western blot analysis for caspase 7, caspase 3, and poly(ADP)-ribose polymerase. We found that co-treatment of cells with SAHA and enzastaurin resulted in caspase and PARP cleavage that was markedly increased compared with single agent treatment (Figure 2). FAS-L also was increased in treated cells, suggesting that up-regulation of this pro-apoptotic molecule may be involved in apoptosis induction and activation of the caspase cascade (data not shown).

Next, we examined a possible effect of the combination treatment on cell cycle progression. Due to the different sensitivities of SU-DHL-6 and OCI-LY-19 to enzastaurin and SAHA treatments, we observed different trends in cell cycle modulation, especially changes in S and G2/M phase populations (Figure 3). In SU-DHL-6 cells, 24-hour enzastaurin single treatments were accompanied by a decreased number of cells within S and G2/M phase, while in OCI-LY-19 cells enzastaurin induced an increase of G2/M phase cell population without any changes in the percentage of cells in S phase. SAHA treatment had no effect on the SU-DHL-6 cell cycle, but in OCI-LY-19 cells it decreased the number of cells within S phase and induced a slight G2/M arrest. Cell cycle changes in both cell lines after the combined treatment exhibited trends similar to those in enzastaurin single treatment. However, these were accompanied by a significant decrease in the number of cells within G0/G1 and by G2/M accumulation.

Figure 2. Western blot analysis of caspase-7, caspase-3, and PARP cleavage, in treated SU-DHL-6 and OCI-LY-19 cells.

Figure 3. Flow cytometric analysis of the cell cycle of SU-DHL-6 and OCI-LY-19 cells treated with 1 and 2.5 µM enzastaurin alone and in combination with 0.5 µM SAHA for 24 hrs.
Having demonstrated synergistic apoptosis induction and cell cycle arrest in B-cell lymphoma cell lines, we expanded our study to other lymphoma cell lines and confirmed a similar finding in ABC-like lymphoma lines OCI-LY-3 and OCI-LY-10 lines, T-cell lymphoma lines, and primary cells from T-cell leukemia/lymphoma (3) and B-cell lymphoma (2). We confirmed apoptosis induction in all samples upon co-treatment with enzastaurin and SAHA and synergistic effect of the two agents. Representative results are shown in Figure 4.

The molecular events underlying the synergistic effect using HDACi with enzastaurin are yet to be fully elucidated. However, our data suggest that PKC inhibitors in combination with HDACi have potential therapeutic value in lymphoma treatment.

References

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HEA Assay: Red Cell Blood Group Antigen Typing by DNA Microarray

By Suneeti Sapatnekar, MD, PhD

Introduction
Testing red cells for multiple blood group antigens is helpful in red cell alloantibody evaluations and in selecting antigen-matched red cell units for transfusion. A serological assay using antisera specific for the antigens of interest is the standard method for typing. However, serological typing is laborious, and commercial antisera are expensive, often in short supply and are not available for some clinically significant antigens. Moreover, serological typing is not suitable for patients with a positive direct antiglobulin test (DAT) or recently transfused patients. Clearly, an alternative method to address these limitations is needed, and DNA-based testing provides a viable solution (1-3).

Microarray Technology
For many red cell antigens (notably excluding the ABO and D antigens), alleleic differences generally result from single nucleotide polymorphisms (SNPs); detecting these SNPs provides the basis for antigen typing. Peer-reviewed data are available for several assays based on this principle, but most are not commercially available in the United States at present. Currently, the most widely used assay is the HEA BeadChip™ assay (BioArray Solutions, Warren, NJ). This assay has been used successfully for antigen typing of blood donors and patients, including patients with complex immunohematological presentations following transfusion or transplantation (4-9).

The HEA assay tests for SNPs associated with the following red cell antigens: C/c, E/e, K/k, Js/Js, Kp+/Kp-, Fya/Fyb, GATA (silencing FY), Fy-a (FY(b+)), Jk+a/Jk-b, M/N, S/s, silencing S (x2), Lu-a/Lu-b, Dvo/Dvo, Hy+/Hy-, Jo(a+)/Jo(a-), LW-/LW, Di-a, Di-b, Cov/Cov, Sc1/Sc2. In addition, the assay tests for the presence of the HbS mutation (4,10).

The HEA assay usually is performed on DNA extracted from EDTA whole blood, but other sources of DNA (e.g. buccal smears) may be used. The DNA region of interest is amplified by multiplex PCR and hybridized with allele-specific oligonucleotide probes for the relevant SNPs. The hybridization reaction is performed on a 300 x 300 μm semiconductor chip mounted on a slide. Each chip contains beads expressing a library of probes matching either the wild-type or mutant allele. Hybridization of the test DNA with a matching probe results in probe elongation that is visualized by incorporation of a fluorescent label. An imaging system analyzes the fluorescent signal intensities for each SNP.

Results Interpretation
The HEA assay software compares signal intensity on the microarray to the location of specific paired alleles and uses this information to determine the presence or absence of each allele and the probable antigen phenotype. Each allele is listed as “+” (present) or “0” (absent). Occasionally, an allele is not reportable due to problems with the signal intensity, and testing may be repeated to obtain valid results.

DNA-based testing may be used to type recently transfused patients, and valid results have been reported even for massively transfused patients. Results should be interpreted cautiously for patients transfused with non-leukoreduced blood, infants, hematopoietic stem cell transplant recipients, immunocompromised patients, and patients with leukopenia or leukocytosis (11-13). Assay results may occasionally be discrepant with the expressed phenotype, and such discrepancies must be investigated (14).

Advantages and Limitations
The HEA assay overcomes the previously listed limitations of serological testing i.e. availability of typing antisera and suitability of the patient sample. Another attractive feature of the assay is that typing for a large number of antigens as well as HbS status can be performed on a 200 μL blood sample.

The HEA assay does not detect all clinically significant antigens; the gene of interest must have been cloned and characterized to be detectable by molecular methods. Also, not all polymorphisms responsible for allelic variants are known, and polymorphisms that are not represented in the assay will cause a false negative result. This is a particular concern for samples from minority ethnic populations.

The HEA assay is not yet licensed by the FDA and is available for research use only.
**Test Validation**

The HEA assay was validated by the Cleveland Clinic Transfusion Service according to the AABB Standards for molecular typing of red cell antigens (15). Twenty-two blood samples were tested, comprising four non-patients, two patients with positive DAT, five patients transfused within three days and 11 other patients, including one with sickle cell anemia.

Reportable results were obtained on all samples, and the results were identical with reference HEA assay results performed at an independent laboratory. Repeat testing by two different technologists resulted in excellent reproducibility. Occasional non-reportable alleles occurred, but these accounted for <0.7% of all alleles, well within the limit of 5% defined by the validation plan.

The sample results correlated well with the available serological and Sickledex testing, except for a single discrepancy for the C antigen (positive on serological testing but negative on the HEA assay). The HEA assay identified this sample as a possible r's allele, a hybrid RHD/RHCE gene that results in a partial C antigen (16, 17). Patients with this variant may test positive or negative on serological testing for C antigen. Further resolution of the discrepancy was not pursued as the patient is deceased.

**Clinical Application**

The HEA assay is indicated as an adjunct to standard pretransfusion testing when a red cell antigen type is needed but serological testing cannot be performed. Appropriate candidates for the assay include:

1. Recently transfused patients

![Polymorphism](image.png)

Figure: HEA assay results on sample from a 66 year-old African-American female with anti-E, anti-C, anti-Cw, anti-Jkb, and additional reactivity without identified specificity. Frequently transfused African-American patients have a high risk of responding to red cell transfusions with alloantibody formation because of the antigen mismatch between the patient and the predominantly Caucasian donor population. In this case, red cell typing was indicated for selecting donor units matched for the patient’s type, but standard serological typing could not be performed because of positive DAT. (a) Phenotype results indicate antigens not expressed on the patient’s red cells, for which donor units may be matched to avoid future antibody formation. (b) Genotype results show that the Fy(b)-negative phenotype is a result of the -33 T>C mutation in the GATA-1 binding motif that prevents Fy(b) expression in red cells, but not in other tissues (see GATA). This patient will not form anti-Fy(b) alloantibody and may be transfused with Fy(b) antigen-positive units, thus expanding the pool of units available for her use and conserving Fy(b)-negative units for other patients.
2. Patients with sickle cell anemia, who are at high risk for red cell alloimmunization (18) and other frequently transfused patients who are noted to readily form red cell alloantibodies when transfused (Figure)

3. Patients with a positive DAT or autoantibodies that interfere with serological testing (Figure)

4. Patients at risk for alloimmunization to the V and Jsα antigens, (typically African-American patients receiving frequent transfusions), as these antigens are rarely represented on standard antibody panels

5. Selected patients with Fyα-negative serological phenotype, for evidence of the Fyα-positive genotype with Fyα silencing mutation, as such patients may not need Fyα-negative red cell units for transfusion (Figure)

6. Patients who need to be typed for high- or low-prevalence antigens for which antisera are not readily available

7. Patients with samples that are insufficient for serological testing.

The HEA assay also can be used to test blood donors to identify antigen-negative donor units for transfusion.

Furture Prospects

A protocol to test samples from leukocyte-reduced donor units is expected to be available shortly. Such a protocol will allow the Transfusion Service to screen donor units and decrease its reliance on the blood supplier to provide antigen-negative units (19).

A kit for the identification of variant antigens in the Rh blood group system also is expected to be available in the near future, and this may be a useful addition to the list of reference tests offered by the Transfusion Service.

BeadChip™ (Illumina, San Diego, Calif.) technology is also available for HLA and platelet antigen typing, and these assays have potential application in the Transfusion Service.

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About the Authors

Eric Hsi, MD, is the Chair of the Department of Clinical Pathology in the Institute of Pathology & Laboratory Medicine.

A graduate of the University of Michigan School of Medicine, he completed a residency in pathology and a fellowship in surgical pathology at the University of Michigan Health System hospitals.

Dr. Hsi is board-certified in anatomic pathology, clinical pathology and hematopathology. His specialty interests include leukemia and lymphoma, immunophenotyping, molecular diagnostics and the development of new therapeutics.

Dr. Hsi can be contacted at hsie@ccf.org or 216.444.5230.

Suneeti Sapatnekar, MD, PhD, is a clinical associate in the Department of Clinical Pathology.

Dr. Sapatnekar earned her medical degree from the University of Poona-Byramjee Jeejeebhoy Medical College in Maharashtra, India followed by a doctorate in biomedical sciences at Case Western Reserve University in Cleveland, Ohio. She served a residency in pathology at University Hospitals of Cleveland and a fellowship in blood banking and transfusion medicine with the Greater Cleveland Chapter of the American Red Cross.

She can be contacted at sapatns@ccf.org or 216.444.3508.