Predicting response to therapy with BH3 profiling

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June 2020—Precision medicine in oncology, which today is nearly universally about genetics, needs to move beyond omics and static approaches, Anthony Letai, MD, PhD, professor of medicine at Dana-Farber Cancer Institute and Harvard Medical School, said at last year's meeting of the Association for Molecular Pathology.

Dr. Letai reported how his laboratory uses dynamic BH3 profiling, a novel assay that detects BCL2 protein dependence in cancer cells and measures changes in their apoptotic priming, to predict clinical response to therapy. He and others call it functional precision medicine.

"The fundamental part of what we call functional precision medicine is taking the drugs you're interested in and putting them in contact with the tumors you're interested in and measuring something fast and something relevant. We choose to use BH3 profiling." Others doing the same work use different methods, he said.

"But the fundamental change is that we realize the incredible value of exposing actual patient samples to drugs, and the technology now exists to do this in a rigorous way."

"There is an enormous amount of actionable information that can be obtained from taking the actual cancer cell you're interested in and subjecting it to a relevant perturbation, that is, exposing it to the actual drugs," he said. "We nearly completely overlook this in today's precision medicine approaches, and I think there is enormous unrealized potential in this general approach."

Dr. Letai

Uniting these efforts worldwide is what led he and colleagues to form the Society for Functional Precision Medicine, of which he is president, and they need the pathology community, he said. "In order for these functional approaches to penetrate more, we have to think twice before we immediately kill the samples [with formalin]. As soon as we kill these samples, the ability to gain functional information is lost," along with the ability to choose the drugs that will work in cancer patients.

The phenotype of increased apoptotic priming is likely to be the main reason chemotherapy ever works, he said. "It can be very useful in assigning this exciting new class of drugs called BH3 mimetics, of which venetoclax is the first member to gain FDA approval" for chronic lymphocytic leukemia and acute myeloid leukemia. The work of his laboratory sped those approvals.

"If you were waiting for genetics to tell you that you should use venetoclax in CLL or AML—a very effective drug in these two diseases—you would still be waiting. There are no mutations, no copy number variations, in either of these two diseases in BCL2 or in related genes. It is not related to genetics; it is related to a functional phenotype to which genetics is completely blind," he said.

"Help us. Join us," Dr. Letai urged the molecular pathologists at the meeting. "The pathologists are the ones who know how to do these tests, who know how to convert an interesting laboratory assay into something that can be used in the clinic, that is rigorous enough analytically that it can be reproducible" and used to make patient treatment decisions. He credits pathologists Annette Kim, MD, PhD, and Fabienne Lucas, MD, PhD, of Brigham and Women's Hospital, in "making us think a little more like clinical pathologists in understanding what it takes to take

an interesting laboratory finding and turn it into a rigorous laboratory test."

His laboratory has focused on the interaction of BH3 peptides with the BCL2 (B-cell-lymphoma-2) family of proteins, of which there are pro- and antiapoptotic members, and their roles in apoptosis. "The point of commitment to programmed cell death is that event of permeabilization of the mitochondrial outer membrane, which is regulated by the BCL2 family of proteins," he said.

Cell damage signals can prompt BH3 activator peptides Bid and Bim to activate the proapoptotic members of the BCL2 family, Bax and Bak, causing them to homo-oligomerize at the mitochondrial outer membrane, resulting in permeabilization and cell death. The antiapoptotic BCL2 molecules can bind to and sequester the proapoptotic molecules to thwart interaction and prevent permeabilization.

"How can we use this knowledge to figure out how close a cell is to the threshold of apoptosis?" Dr. Letai said. His original plan to measure all the proteins and total them up in "some weird equation" was too complicated.

"Instead we took a more simple and functional approach, which is we synthesized these BH3 peptides," he said. Drawing on his postdoctoral work in the laboratory of the late Stanley J. Korsmeyer, MD, Dr. Letai showed that the synthetic BH3 peptides "are essentially equivalents of the proapoptotic proteins" and can be easily measured.

This led to the next question: "How much BH3 peptide do we need to add until the mitochondrion permeabilizes?" A cell with a mitochondrion that required a large amount of BH3 peptide for permeabilization was considered far from the threshold and unprimed for apoptosis. "We simply take mitochondria, expose them to BH3 peptides, and then measure the mitochondrial outer membrane permeabilization," he said.

Dr. Letai described an intracellular-based assay his laboratory developed to measure the effect of BH3 profiling on a single cell suspension of cells, such as leukemia cells. Instead of extracting the mitochondria, "we gently permeabilize the plasma membrane of the cells, so that the peptides that we add can gain access directly to the mitochondria," he said. After 60 to 90 minutes of exposure to Bim BH3 peptides, the cells would be stained with a cytochrome c antibody to measure cytochrome c release from the mitochondria (Ryan J, et al. *Methods.* 2013; 61[2]:156–164).

"Note that the cytochrome c is an intermembrane space protein, so when the outer membrane is ruptured, it leaves the mitochondria," Dr. Letai said. The cell membrane permeabilization results in a cytochrome c-negative cell.

"Why do we care?" he said.

His laboratory first used BH3 profiling to understand BCL2-inhibitor drugs and which diseases to target with venetoclax (ABT-199), the latest version of the BCL2-inhibitor drugs. "It is a great [BCL2] antagonist, very selective, very potent." And the question is, for what diseases can it be used?

Dr. Letai's team used fluorescence polarization binding assays to determine the sensitivity of BH3 peptides to the BCL2 antiapoptotic proteins. "Certain peptides are promiscuous but some are very selective. For instance, the HRK BH3 peptide selectively binds and inhibits BCL-XL," he said. A cell that readily permeabilizes its mitochondria in response to the HRK peptide, for example, is a BCL-XL-dependent cell, while the Noxa BH3 peptide can be used to identify MCL1-dependent cells. The Bad BH3 peptide is selective for BCL2-dependent cells (Certo M, et al. *Cancer Cell.* 2006;9[5]:351–365).

They tested the theory in model systems of known BCL2 and MCL1 dependents, and then turned their attention to human cancers, "to diseases found in the wild," Dr. Letai said. They looked first at CLL, for which there were discrepancies in the literature about the apoptotic protein dependence of CLL. "And this pretty much puts it to rest. The mitochondria of CLL patients are uniformly sensitive to the Bad peptide but not to the HRK peptide," which is diagnostic of BCL2 dependence, he said. "When we exposed CLL cells to ABT-737, a primordial BCL2 inhibitor, it was universally sensitive to the inhibitor" (Del Gaizo Moore V, et al. *J Clin Invest.* 2007;117[1]:112–121).

Dr. Letai's team further showed that introducing a BCL2 inhibitor, such as ABT-737, to CLL cells quickly caused the displacement of proapoptotic proteins and the activation of the proapoptotic protein Bax to induce oligomerization and cell permeabilization. "Within hours you could see the normalization of the white blood count in the periphery," he said. "That's how rapidly this effect happens."

Similar work with AML myeloblasts showed BCL2 dependence in AML, though "we ran into enormous skepticism," Dr. Letai said. "People just didn't believe that BCL2 could be involved in a myeloid malignancy."

His team's work showed that most AML myeloblast samples were more sensitive to the BH3 Bad peptide, and thus more BCL2 dependent, than normal hematopoietic stem cells. "This suggests that there was already built in for us a therapeutic index for BCL2 inhibition in AML," Dr. Letai said.

At this point, Dr. Letai connected and shared data with Marina Konopleva, MD, PhD, a physician-scientist and professor in the departments of leukemia and stem cell transplantation at MD Anderson Cancer Center, and they presented the data to Abbvie "and convinced them we should start a clinical program in AML with BCL2 inhibition, because the preclinical work was very strong," he said.

The first human trial of venetoclax in AML patients showed "definite evidence of clinical activity of BCL2 antagonism in the case of AML," Dr. Letai said. He and Dr. Konopleva reported an objective response rate of 19 percent in the trial patients, the majority of whom were elderly and had received at least one prior therapy (Konopleva M, et al. *Cancer Discov.* 2016;6[10]:1106–1117).

In subsequent trials of venetoclax with hypomethylating agents Vidaza (azacitidine) or decitabine, Dr. Letai said, "responses were remarkable. In treatment-naïve elderly patients, the CR/CRi [complete remission/complete remission with incomplete marrow recovery] is upward of 70 to 75 percent. Just a remarkable achievement with an induction regimen that is essentially taken at home" (DiNardo CD, et al. *Blood.* 2019;133[1]:7–17).

When BCL2 inhibitors made it into the clinic for CLL, he said, "it was a remarkable result—79 percent response rate with complete remission in 20 percent of patients" (Roberts AW, et al. *N Engl J Med.* 2016;374[4]:311–322).

"Now there are FDA approvals in CLL and AML, in a variety of settings, for venetoclax in the United States and around the world," Dr. Letai said.

What about general chemosensitivity to conventional chemotherapy agents?

"It's a bit of a mystery why these should selectively kill cancer cells," Dr. Letai said. "So we tested the hypothesis that perhaps these types of drugs perform damage and cause apoptotic signaling in every cell in our body, but there are some cells we'll call primed cells, that are right on the edge." These primed cells respond to apoptotic signaling by going over the threshold and committing to apoptosis, "whereas less primed cells, like chemoresistant cancer cells or most normal cells, when subjected to these death insults, move toward the cliff but not over it."

Cue the promiscuous BH3 peptides. Bim, Bid, and Puma interact with all of the antiapoptotic proteins and are good measures of net apoptotic reserve—"basically, how far you are from the edge of the cliff."

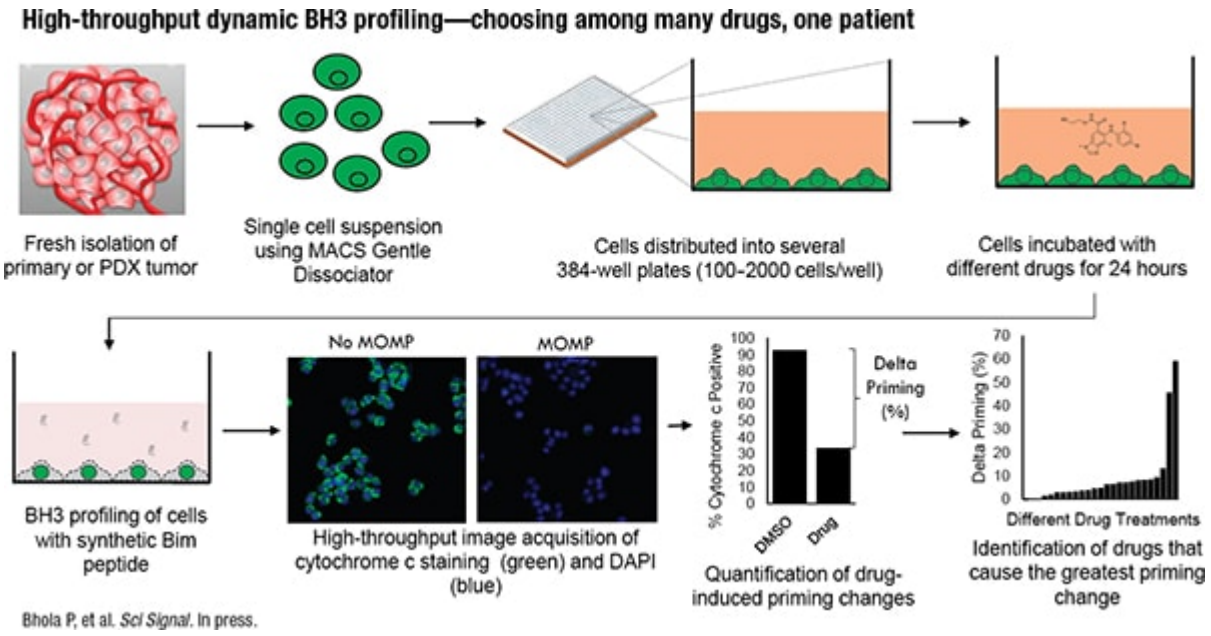
"We asked, 'Does sensitivity to these peptides at the mitochondrial level correlate with sensitivity at the clinical level to these conventional chemotherapeutic agents?'" he said. "And it turns out it did."

For example, results of studies of patients with either multiple myeloma, ALL, or ovarian cancer found that patients who responded well to standard clinical therapies were more primed at the mitochondrial level than patients who responded poorly (Ni Chonghaile T, et al. *Science.* 2011;334[6059]:1129–1133).

"We have since found this in many different diseases," Dr. Letai said. Examinations of normal chemotherapy-resistant tissue, such as the heart, lung, and brain, find these tissue cells tend to be significantly less primed for apoptosis. The unprimed tissue in the adult body "is the main reason why there is a therapeutic index for chemotherapy, and why we can kill blood cancers but we can't, in general, cure solid tumors with chemotherapy.

Blood cancers and normal blood cells are the most primed cells in our body."

The theory held true during his team's study of myeloblasts with a comparison of AML patient responses to conventional chemotherapy—stable complete remission, complete remission that relapsed, or poor response. "It correlated very well with how primed their mitochondria were," as measured by response to the Bim peptide, he said. AML patients with highly primed mitochondria achieved better outcomes; those patients with myeloblasts that were more primed than normal hematopoietic stem cells were found to stand a better chance of cure with chemotherapy alone.



What can be done for unprimed patients? "Maybe we could make unprimed cancer cells more primed," Dr. Letai said. "Are there ways we could expose tumors to chemotherapies and see whether they evoke apoptotic signaling ex vivo, in the lab, using primary patient tumor cells?"

Dr. Letai and his team found that their dynamic BH3 profiling technique could be used ex vivo to predict the cytotoxic response of cancer cells to various treatments. "Turns out our tool could do this because the induction, the initiation of apoptotic signaling, happens within hours. And that means we do not demand long-term ex vivo culture for our primary cancer cells," he said. "This has long been the hang-up of ex vivo approaches to measuring chemotherapy sensitivity in cancer" (Montero J, et al. *Cell.* 2015;160[5]:977–989).

"Now we have a way." His laboratory takes liquid or solid cancer cells, turns them into a single-cell suspension, distributes them to 384-well plates, then exposes the cells to treatments. Next they measure: After six to 24 hours of exposure to the drug, what is the apoptotic priming of the cancer cells compared with a well containing untreated cancer cells? "That's what we call our delta priming," Dr. Letai said, "and we have subsequently found that the bigger the delta priming, the better the in vivo response."

Does it work? In one trial, 21 different B-cell acute lymphoblastic leukemia patient-derived xenografts (PDX) in mice were exposed to the HDM2 (MDM2) inhibitor CGM097. The results: "We can, with perfection in this relatively small number of samples, completely discriminate the responders from the nonresponders based on this ex vivo assay," Dr. Letai said (Townsend EC, et al. *Cancer Cell.* 2016;29[4]:574–586).

A human study using dynamic BH3 profiling to predict imatinib response in CML patients also had encouraging results. "We were able to discriminate, not with the same perfection but still quite well, between those who

responded well and those who responded poorly to imatinib in the clinic."

The next step, Dr. Letai said, would be to identify one therapy among several choices that would work best in a particular patient. Using AML PDX models, his laboratory performed dynamic BH3 profiling with a set of molecules and then measured the in vivo response to four different types of single-agent therapies: birinapant (second mitochondria-derived activator of caspase mimetic), quizartinib (FLT3 inhibitor), JQ1 (BDR4 inhibitor), and venetoclax.

"In each of these cases, our assay predicts very well the magnitude of the in vivo response, suggesting that across many different drugs we can continually predict the in vivo response," Dr. Letai said of the unpublished study results.

"We can do this in the clinic," he said. In a clinical trial with cohorts at Stanford Medicine and Massachusetts General Hospital, Dr. Letai's team used dynamic BH3 profiling to evaluate AML patient response to treatment with lenalidomide plus chemotherapy with mitoxantrone, etoposide, and cytarabine. By studying the patient myeloblasts prior to therapy, exposing them ex vivo to lenalidomide and MEC, and measuring the delta priming, Dr. Letai's laboratory was able to predict with good precision the patient response (Garcia JS, et al. *Am J Hematol.* 2020;95[3]:245–250).

"This works also in solid tumors," Dr. Letai said. A high throughput automated microscopic assay developed in his laboratory enables testing for thousands of drugs using the same sample, provided there are enough cells to distribute to the different wells. "We can do primary discovery directly on patient primary tissues in addition to smaller, clinical diagnostic studies" (Bhola P, et al. *Sci Signal.* In press.).

This assay uses immunofluorescence and automated analysis to measure the release of cytochrome c. Comparing the amount of cytochrome c positive cells in drug-treated versus untreated wells informs the evaluation of whether the drugs affect the sensitivity of the mitochondria to the promiscuous BH3 Bim peptide. "We get different delta primings for each drug and rank the quality of the drugs on that basis."

Confident in their ability to discriminate between active and inactive compounds, Dr. Letai and his colleagues took an in vivo tumor model, MMTV-PyMT (mouse mammary tumor virus-polyoma middle tumor-antigen), and tested it across a 1,600-member bioactive library.

"Our question was, 'Can we pick the drugs that will cause remission to these breast tumors by purely functional means?'"

While most of the 1,600 drugs "did nothing, which is great," Dr. Letai said, his laboratory identified and prioritized for testing the drugs that selectively caused apoptotic signaling in the tumor cells but not the normal cells. "We chose a handful of positive and negative controls to see if we could predict the ones that would work."

They tested two drugs for mouse breast tumor regression—an HSP90 inhibitor and a BCR-ABL kinase inhibitor—as single agents and in combination therapy. After 14 days of treatment with the drug combination, "we essentially had a complete remission of these murine breast tumors."

Although removal of the two agents resulted in tumor regrowth, "it's still strong proof that we're able to identify active drugs that can be combined into effective combination regimens," Dr. Letai said. A more comprehensive view of testing of drugs they predicted would work and not work showed "good correlation between what we measured in terms of delta priming and what was observed in terms of treatment of the actual mouse."

Dr. Letai said the BH3 profiling assay is not far from being able to be performed in a CLIA-certified laboratory, thanks to the expertise of pathologists Dr. Kim and Dr. Lucas. Once it is, he said, it should be transferable. "There's no reason why it wouldn't be transferable to another center with proper training. We don't do any magic. These are recipes, cookbooks, protocols that could be performed by a cell biologist."

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