Biosynthetic gene clusters for two enediynes have been identified, and an enediyne cancer drug is being tested

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Remember the enediynes? These bacterial natural products, first isolated in the 1960s, were found to have powerful antibiotic and anticancer properties. In the late 1980s and early '90s, they generated intense research interest as organic chemists competed to be first to synthesize them and drug companies pursued potential enediyne drugs.

Enediyne research has indeed continued to perk quietly along, and several lines of investigation are now coming to fruition: The production genes for two enediyne have just been identified, a new resistance protein (a protein that protects a producing organism from its own enediyne) has been found, enediyne clinical trials are being carried out, and an enediyne-based antibody conjugate is currently up for marketing approval.

The shared feature of all enediyne compounds is their enediyne chemical group—a large carbon ring containing two triple bonds separated by a double bond. The enediyne group is often called a warhead because it's more than ready to cyclize, forming benzene via a highly reactive 1,4-benzenoid diradical intermediate. The diradical is the species responsible for oxidative DNA cleavage and the enediyne's cell-killing ability. This cyclization process, named the Bergman cycloaromatization reaction, was discovered in 1972 by chemistry professor Robert G. Bergman of the University of California, Berkeley.

The identification and characterization of one set of enediyne biosynthetic production genes—those of the enediyne C-1027—were carried out by graduate student Wen Liu and assistant professor of chemistry Ben Shen of the University of California, Davis [Antimicrob. Agents Chemother., 44, 382 (2000)]. "We cloned the genes and demonstrated that we can improve the yield by manipulating the biosynthetic pathway," Shen says.
Shen

The significance of having C-1027's gene cluster in hand is that "genetic engineering of biosynthetic pathways can lead to discovery of new compounds that are difficult to make by conventional chemical synthesis, and some can be better drugs," Shen says. "Using genetic engineering, you can also add extra copies of activators to the pathway to improve the yield of natural product. People normally do random mutagenesis to identify high producers—a tedious, painstaking process called strain improvement—but, having the gene cluster, we can now do that rationally and do it much faster."

Calicheamicin resistance protein discoverers Whitwaw

In C-1027 and other chromoprotein enediyines (enediyines associated with an apoprotein during biosynthesis), the protein wraps around the enediyne, sequesters and stabilizes it, and shepherds it out of the cell, thus protecting the producing bacterium from potentially lethal exposure to the enediyne's toxic fury.

Medicinal chemistry professor C. Richard Hutchinson of the University of Wisconsin, Madison, who specializes in the biochemistry and genetics of antibiotic biosynthesis, comments that "it's always been fascinating to think about how the protein and molecule are made inside the cell and assembled. Now we have ways to look at that—access to the genes and mutants that might be made to try to probe that novel metabolite-protein packaging problem."
At the same time, Jon S. Thorson, an assistant member of the molecular pharmacology and therapeutics program at Memorial Sloan-Kettering Cancer Center, New York City, and coworkers have discovered and characterized the resistance protein for the enediyne calicheamicin and have identified the calicheamicin gene cluster [J. Am. Chem. Soc., 122, 1556 (2000)]. A resistance protein serves to protect a bacterium from enediyne it produces biosynthetically—so it is not hoisted on its own enediyne petard, so to speak.

Thorson's coauthors were postdoctoral fellows Ross E. Whitwam and Joachim Ahlert; assistant professor of chemistry and biochemistry Theodore R. Holman of the University of California, Santa Cruz; and Mark Ruppen of the bioprocessing development group at the Wyeth-Ayerst Research Division of American Home Products, Pearl River, N.Y. (Whitwam is currently assistant professor of biology at Mississippi University for Women, Columbus.)

If bacteria that produce enediyne must be protected somehow from their own natural products, why do they produce such toxic compounds in the first place? "Most of these bacteria are very slow growing," Thorson explains, "and can therefore be easily overcome by other organisms. The toxic metabolite is used to build a lethal 'moat' around the producing strain to keep other organisms from invading its space."

Calicheamicin is an incredibly potent antibiotic and antitumor agent—more than 1,000 times more potent than Adriamycin, one of the most effective cancer drugs in clinical use. So, protecting the producing microorganism from calicheamicin is a nontrivial task. The chemical role of the resistance protein is to quench the calicheamicin benzene-1,4-diradical intermediate that forms when the enediyne warhead is triggered. Up to now, nobody has known how the calicheamicin-producing bacterium controls the toxic effects of this highly reactive substance.

Thorson and coworkers have now identified a resistance protein called CalC that is responsible for this protective effect, and they have carried out preliminary studies on its mechanism of action. The resistance mechanism is still unclear, but it seems to be of a type not previously observed.
Thorson speculates that the calicheamicin molecule is normally sequestered throughout its biosynthesis and is then quickly pumped out of the cell. "We've come across a number of genes coding for membrane transporters in our cluster, which supports that view," he says. If a little bit of calicheamicin escapes the pumping mechanism and gets into the cytosol of the bacterial cell, "my belief is that CalC inactivates calicheamicin before it does any harm to the bacterium—although it may do more than that."

His group has demonstrated that CalC requires an active-site nonheme iron for activity, suggesting that it's an enzyme with a novel electron-transfer relay mechanism. "But there are still some key experiments required to support or refute that possibility," Thorson says.

The researchers do know that CalC is a completely novel protein, in that a homology search against sequence databases reveals no matches. Because its sequence is unlike that of any other protein, the protein's mechanism may likewise prove to be novel.

The identification and localization of CalC helps confirm the discovery of the calicheamicin biosynthetic gene cluster, Thorson says. "Nowadays, part of the formal proof for cloning a gene cluster for a secondary metabolite like calicheamicin is whether or not you have a resistance gene within that cluster for that particular metabolite. CalC definitely gives resistance to calicheamicin both in vivo and in vitro, and it's found within a cluster of genes that are consistent with other proposed steps in the biosynthesis of calicheamicin."

Thorson says he and his coworkers "use the CalC discovery as part of our formal proof that the calicheamicin cluster has been cloned, and we have additional biochemical support for a number of our biosynthetic gene assignments." Their first published biochemical evidence was reported last year, when Ahlert and Thorson used a calicheamicin gene to make hybrid antibiotics, in a combinatorial biosynthesis study carried out in collaboration with professors Hung-wen (Ben) Liu and David H. Sherman of the University of Minnesota, Minneapolis, and coworkers [J. Am. Chem. Soc., 121, 9881 (1999); C&EN, Nov. 1, 1999, page 26]. So the calicheamicin biosynthetic genes have already been used to make new molecular variants.
Currently, Thorson's group is collaborating with others in efforts to express CalC in eukaryotes. "Should calicheamicin toxicity ever become an issue in therapy, one could then imagine intervening with CalC," he says.

Hutchinson says: "It's an amazing coincidence that two labs have gotten to a similar point, but with completely complementary pathways. They certainly have broken open what has been a long-time intriguing problem. However, the real excitement will come when they and others follow the road they've started on. They both have characterized at least some of the sequence and enough of the flanking open reading frames to know that they have the biosynthetic genes, without question. That's going to awaken a lot of interest in the biochemistry of how nature puts these things together and how that might be manipulated eventually to create a few variations in the structure by biological methods" as a route to new drug candidates.

Preliminary studies by the Shen and Thorson groups already provide intriguing hints about the biosynthesis of C-1027 and calicheamicin. There are two ideas about how the carbon skeletons of enediyynes are assembled. One hypothesis is that they are made from long-chain unsaturated fatty acid precursors, such as oleic acid or linoleic acid, and the other is that they are assembled by a polyketide synthase from acetic acid or malonic acid precursors.

So far, Thorson and coworkers have found genetic sequences that fit the polyketide synthase paradigm in the calicheamicin biosynthetic gene cluster. However, Shen is less certain that the mechanism of the C-1027 biosynthetic gene cluster fits neatly into one of the two proposed classes. "We have the entire cluster, and if the enediyne is assembled by a polyketide synthase, we definitely will not miss it within the cloned gene cluster," Shen says. "But the microorganism is not using known chemistry to make this compound."

Thorson notes that "the ability to compare and contrast the C-1027 and calicheamicin pathways should shed light on what appears to be a significant divergence from the typical fatty acid or polyketide paradigm. Ben and I are both very excited about the implications."

Of all the enediyynes, only neocarzinostatin is approved right now for clinical use, and that's only in Japan. It's marketed as part of a protein-polymer drug called SMANCS that is used to treat liver cancer.

Among the other enediyynes, calicheamicin seems to be heading most directly toward the clinic. Calicheamicin's unusual chemistry and potent antitumor properties were studied in the 1980s at American Cyanamid's Lederle Laboratories, now the Wyeth-Ayerst Research Division of American Home Products. Subsequent fermentation improvements and efforts to attach calicheamicin to various monoclonal antibodies led to drug-antibody conjugates suitable for clinical trials. Such a conjugate, in which calicheamicin is linked to a monoclonal antibody designed to direct it specifically toward tumor cells, recently passed a Phase II trial for acute myeloid leukemia (AML). The conjugate is being developed by Wyeth-Ayerst Laboratories, Radnor, Pa., and Celltech Chiroscience, Slough, England.
AML is a fatal disease characterized by an accumulation of abnormal white blood cells in the blood and bone marrow, resulting in anemia, infection, and hemorrhage. The AML drug conjugate, trade named Mylotarg (and called gemtuzumab ozogamicin generically), is a humanized recombinant antibody that is linked to calicheamicin. The antibody is specific for the CD33 antigen, a protein commonly expressed by myeloid leukemic cells. CD33 is also expressed by some normal blood cells, and Mylotarg has some side effects that may stem from its impact on those nontarget cells. However, the normal hematopoietic stem cells that produce new blood cells do not express CD33 and are not targeted by Mylotarg, so normal blood cells that are damaged can potentially be replenished.

In the drug Mylotarg, a potential treatment for acute myeloid leukemia, two calicheamicin molecules (lower left and right) are conjugated with a monoclonal antibody specific for CD33 receptors found on surfaces of myeloid cells and some normal blood cells. [© 1999 Wyeth-Ayerst]

In an earlier Phase I study of the drug in patients with relapsed or refractory AML, "it was really quite well tolerated, and we were pleased to see several patients enter remission using the agent, which then led to Phase II studies," explains oncologist Eric Sievers of Fred Hutchinson Cancer Research Center, in Seattle, whose group carried out the trials.

In the Phase II study, Sievers and coworkers treated 59 patients with the agent. "All of these patients were in a first untreated recurrence of their leukemia after being in remission. Our response rate was about 34% in that group. That meant that it eliminated their leukemia and that their blood counts were returning to reasonably normal levels. For a single agent for this disease in patients who have their leukemia coming back, this is a very reasonable response rate."

An appealing aspect of the calicheamicin treatment, Sievers says, "is that it's not associated with some of the very classic toxicities that we see with standard chemotherapy drugs. Namely, no one lost their hair, and no one was getting mucositis—damage to the gut from the chemotherapy. But we were certainly seeing a drop in blood counts, which was expected from the drug's mode of action, which is to kill cells that express the antigen CD33."

One reason an agent as generally toxic as calicheamicin is fairly well tolerated by patients in these trials, Sievers says, is that "the conjugation with antibody allows us to give only minute quantities to patients. If it goes to the right place, then it achieves its objective, and if it doesn't, it's present in such low quantities that it's not anticipated to have deleterious effects." In addition, he says, "it's our impression that the drug doesn't undergo a rearrangement and expose its free radicals until it is inside a cell. It's thought to kill cells by cutting DNA, causing double-stranded DNA breaks that lead to
Late last year, the Food & Drug Administration assigned priority review status to Wyeth-Ayerst's new-drug application seeking market approval for Mylotarg for the treatment of CD33-positive relapsed AML. According to the company, no chemotherapeutic drugs are currently approved for the specific treatment of patients with relapsed AML. Although Mylotarg has not yet undergone Phase III trials, the application is being considered for approval under regulations that permit new-drug submissions based on Phase II data for oncology and unmet medical needs.

Regarding the potential for using the newly discovered biosynthetic pathways to construct other promising enediyne drug candidates, Thorson says: "Obviously, there is only so far one can go in manipulating the warhead, as chemists have shown even minor structural changes in the warhead can eliminate DNA-cleaving ability. However, the genes of the C-1027 and calicheamicin pathways provide new diversity for the growing toolbox of combinatorial biosynthetic chemists—namely, very novel sugar ligands and triple-bond formation. Exploiting these tools should provide complex hybrid molecules that may be difficult to approach with current synthetic methodology."