

# Nitric Oxide:

## Just say NO to Cancer and Much More

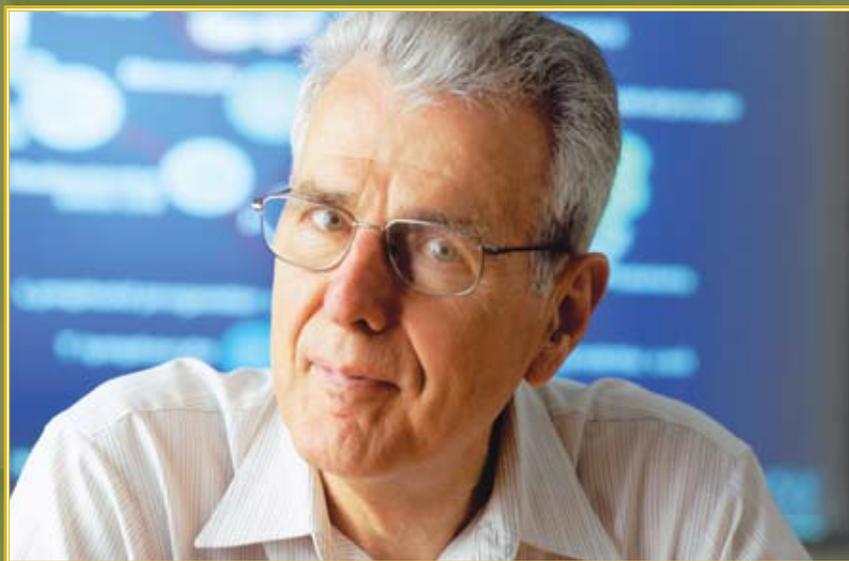
*Nitric oxide (NO)—a simple molecule consisting of one nitrogen atom and one oxygen atom—is everywhere. Blood vessels use it to relax, neurons use it to communicate, and innate immune cells use it to kill dangerous invaders. NO researchers won the Nobel Prize in 1998, barely a decade after its identification as the biological activity known as endothelial-derived relaxing factor (EDRF). As the initially controversial evidence began to accumulate that NO is a key player in several biological processes, Larry Keefer, Ph.D., Chief of CCR's Laboratory of Comparative Carcinogenesis, was ready with the tools to manipulate it for biomedical research. An organic chemist working on NO-related chemistry as a means to understand and ultimately prevent the carcinogenic effects of the nitrosamines found in a variety of foodstuffs, environmental sources, and manufacturing processes, Keefer was poised to jump into the NO fray with the first reliable chemical donor with which to study the effects of authentic NO in culture and in vivo. Since then, he has initiated several collaborations to develop and study agents that can selectively target NO's power to destroy cancerous cells. His goal is to see one of these agents enter the clinic.*

A volatile gas on its own, nitric oxide (NO) is produced where it is needed in the body, and it cannot be directly administered to most biological tissues. Instead, it must be released from other compounds, as in the case of nitroglycerin, which was used to treat heart pain a hundred years before the mechanism producing dilation of blood vessels was shown to be NO.

"My group is [almost entirely] chemists," said Keefer. "We know how to make compounds, make them pure, and design them with specific structural features. That's been our forte. We try to interest collaborators who know how to do the rest of it." One of his most fruitful collaborations has been with University of Utah oncologist Paul Shami, M.D., to study the use of NO to fight cancer.

### NO to Leukemia

Shami demonstrated several years ago that acute myeloid leukemia cells are particularly sensitive to NO toxicity at concentrations of a NO-releasing drug substantially lower



(Photo: R. Eber)

Larry Keefer, Ph.D.

than those that safely maintain normal healthy endothelial or liver cells.

"NO had long been known as a toxic air pollutant, cigarette smoke constituent, and precursor of carcinogenic nitrosamines. But

as its numerous bioeffector roles attest, NO turns out to be essential for proper health just about everywhere in your body," explained Keefer. "So, evolution has provided our cells with ways to deal with its toxic potential."

Shami's leukemia cells had apparently lost some of that ability. This led him to the hypothesis that administering a NO-releasing drug into the general circulation might preferentially eliminate the NO-sensitive leukemia cells without collateral harm to normal tissues. This proved to be the case in a mouse xenograft model of leukemia—a research model in which human cancer cells are grafted under the skin of mice with impaired immune systems to prevent rejection of the foreign graft. The lead compound Shami identified, JS-K, cut the growth rate of the mice's tumors in half without any apparent toxic effects; it also induced more necrotic cell death, relative to controls, in the tumor mass that remained.

Having learned of Shami's success with the *in vivo* leukemia model, Tanyel Kiziltepe, Ph.D., and Kenneth Anderson, M.D., at Harvard Medical School, demonstrated that JS-K also inhibits proliferation of human myeloma cells *in vitro* as well as in xenograft models. Because of JS-K's cell specificity, the doses required to see an effect in mice did not, as expected, cause major changes in vascular tension. The researchers have also studied the mechanisms through which JS-K damages cancer cells and have found evidence for NO-induced DNA damage leading to apoptosis. "I can't put together the whole story on the mechanism yet," noted Keefer. "You look at the structure and chemistry, and there are clearly other pathways by which the compound can be active." Nonetheless, the preclinical evidence is mounting for JS-K's potential as a novel anticancer agent. In a paper published earlier this year in *Leukemia Research*, the team demonstrated that JS-K has a synergistic effect with the antileukemia drug cytarabine in inhibiting proliferation of leukemia cell lines. Shami, in the meantime, has founded a biotechnology company with a confidently optimistic name—JSK Therapeutics.

The efficacy of JS-K appears to extend beyond leukemia and multiple myeloma cells. Similar cytostatic effects have been observed in rodent liver and prostate cancer models. Keefer is also collaborating with Lucy Anderson, Ph.D.,

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Head of the Cellular Pathogenesis Section at CCR, to study JS-K in the multiple human lung cancer cell lines that she and her colleagues have characterized for NO research. Working across the two laboratories, Research Associate Anna Maciag, Ph.D., has unpublished data demonstrating that JS-K is not only effective against lung cancer cells but that it also appears to have an even greater potency in lung cancer cells that have high levels of reactive oxygen species (ROS). "Now we're talking about personalized medicine," commented Keefer. "If a tumor contains high levels of ROS, perhaps it will be an ideal candidate for our drug."

### NO Chemistry, Please

JS-K is actually O<sup>2</sup>-(2, 4-dinitrophenyl) 1-[(4-ethoxycarbonyl) piperazin-1-yl] diazen-1-ium-1, 2-diolate, a chemical name that looks much more complicated than the guiding principle behind its development. Before stepping into the world of NO biology, Keefer and his team had been working on the related chemistry of a widely distributed class of carcinogens called nitrosamines. As interest in the biological effects of NO grew in the late 1980s, Keefer realized that their knowledge of this chemistry might put them in a unique position to contribute to the field. He knew that compounds of a particular structure—XN(O-)N=O where X is any of a variety of molecular groups—could release NO in a controlled fashion and thought that these compounds could be developed for biological applications.

"So, I took a leap," explained Keefer. He had only recently been through the NIH site visit evaluation process, which takes place every four

years, and thus he felt that he could afford to take a chance on this new biology and still turn back if it did not seem fruitful in one or two years.

"The postdoc who started off this work, Tamra Dunams, was very productive," remembered Keefer with respect. "Within the next two years, the NO team we organized including Joseph Hrabie, Chris Maragos, Joseph Saavedra, and David Wink had our first patents, a *Science* paper, and a *Journal of Medicinal Chemistry* paper that is among the most cited in that journal." They tested a series of NO-releasing compounds—diazoniumdiolates or NONOates—and demonstrated that the strength and duration of vasodilation generated by these compounds could be reliably predicted through measurements of their chemical decomposition rates. They then demonstrated a mechanism whereby NO could induce DNA damage by combining with oxygen to disrupt amine groups on DNA directly. "We laid the ground floor for a lot of interesting stuff and are credited with setting worldwide standards for producing reliable fluxes of NO in culture and *in vivo*."

JS-K is just one example of the evolution of that research, and the team is continuing to work on optimizing its composition, chemistry, and delivery. Shami recently presented a study at the American Association of Cancer Research annual meeting describing how JS-K is packaged in lipid nanoparticle micelles to improve its persistence in the bloodstream. Structure-based drug design efforts conducted by glutathione S-transferase (GST) expert Xinhua Ji, Ph.D., of CCR's Biomolecular Structure Section, Macromolecular Crystallography Laboratory, suggested molecular

modifications that he predicted would convert JS-K into a particularly effective substrate for the pi isoform of GST, a protein that is overexpressed in a great many tumor cells. Saavedra incorporated these features into a second-generation compound, PABA/NO. Kenneth Tew, Ph.D., now of the University of South Carolina, and his colleagues studied the mechanisms of PABA/NO's cytotoxicity, confirming the involvement of GST and showing that PABA/NO could slow the growth of human ovarian cancer xenografts in mice with a potency rivaling that of the widely used anti-cancer agent cisplatin.

### NO, Not Just Cancer

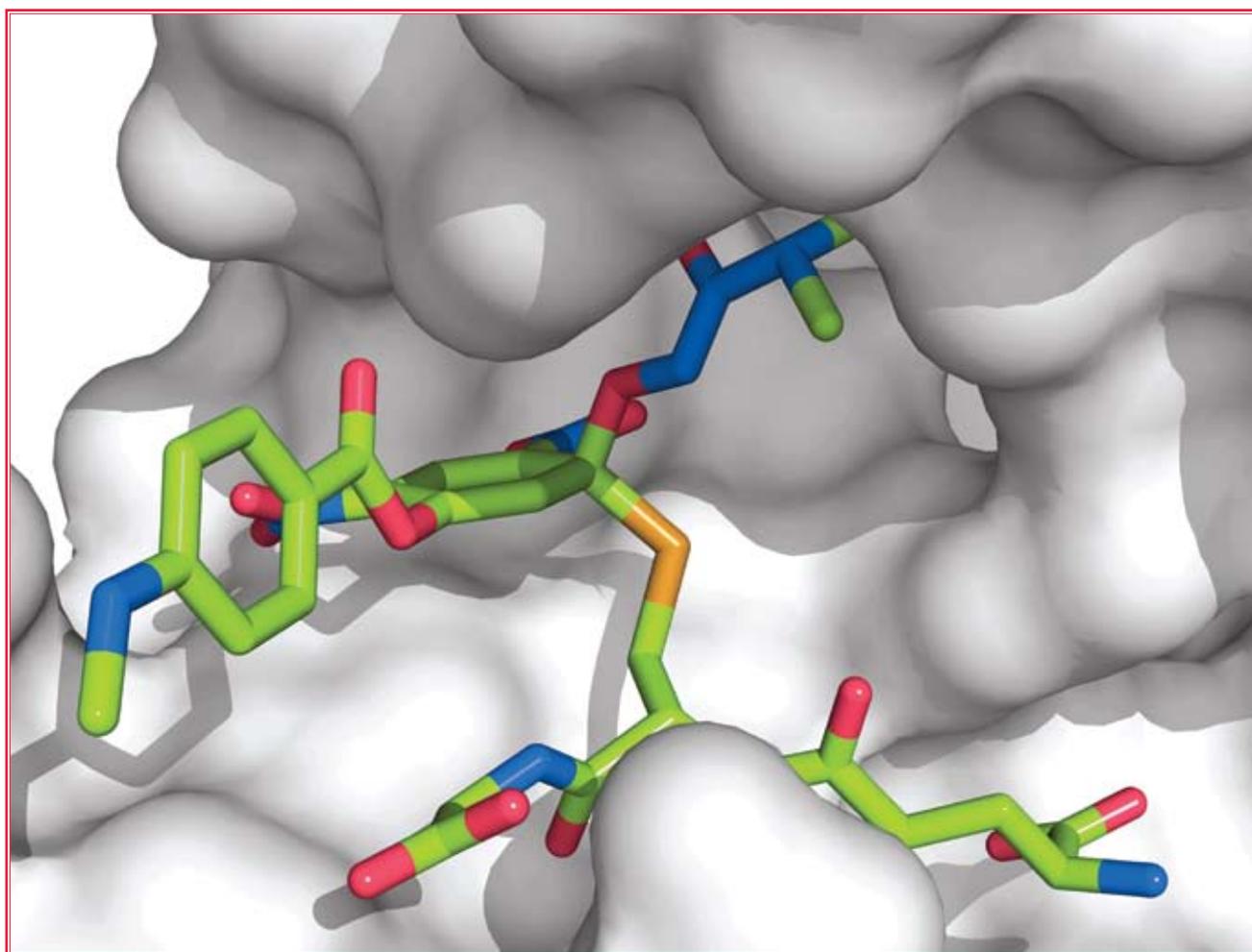
Keefe's early insight that NONOate chemistry might be useful for biomedical

research and the conviction that led him to devote two precious years of his four-year NIH review cycle to a project tangential to his prior work have led him from the world of cancer prevention to cancer therapeutics. However, Keefe is quick to point out that since well-regulated fluxes of NO are key to virtually every biological system, the potential for these drugs goes well beyond cancer. "I'm convinced there are lives to be saved and fortunes to be made based on our technology."

NO can be considered a toxic weapon or a cellular defense. Keefe's laboratory is currently studying NONOates that, in addition to directly attacking cancers, could supplement the NO already produced by macrophages to fight disease. They have also designed compounds that

will release NO only after they have been activated by cytochrome P450, an enzyme found predominantly in the liver. With it, they hope to increase vascular perfusion during liver failure and protect the organ from ischemic damage. They have also studied the use of these NO donors linked to NSAIDs (nonsteroidal anti-inflammatory drugs) as a means of protecting against the gastric ulcers associated with use of NSAIDs alone. The same chemistry can even form the basis of NO-releasing polymers and materials for use in vascular surgery. The applications are, in short, as widespread as NO itself.

To learn more about Dr. Keefe's research, please visit his CCR Web site at <http://ccr.cancer.gov/staff/staff.asp?profileid=5731>.



(Image: Xinfa Li, NCI)

PABA/NO is a NO-donor compound designed to act in tumors overexpressing the enzyme glutathione-S-transferase (GST) pi. GST-pi catalyzes the reaction of PABA/NO with GSH (glutathion) to produce the reaction intermediate shown. The GST active site is illustrated as a molecular surface and the ligand as a stick model in an atomic color scheme (carbon in green, nitrogen in blue, oxygen in red, and sulfur in orange).