The PUZZLE of the PROTEOME

For all of the discoveries made over the past 10 years in proteomics, the primary experimental paradigm of the field remains 2-D gel electrophoresis (2-DE), followed by in-gel protein digestion with a protease such as trypsin, and analysis of the resulting peptides using one of the many flavors of MS. But for all the seeming simplicity of this process, things can go wrong at many stages, and researchers can end up with nothing for their efforts. Thus, academic and corporate scientists have spent a lot of energy in pursuit of methods that simplify or minimize the steps between gel and MS sample plate.

Seeing is believing

One of the main challenges for researchers who perform 2-DE is the ability to visualize each of the protein spots on the gel, especially given the fact that in any sample, one protein can be 10^6-fold more prevalent than another protein, with others falling throughout this range. Thus, sensitive and selective staining methods are needed to efficiently identify the proteins of interest.

One of the traditional methods relies on the interaction of immobilized proteins with silver ions. Silver staining is a particularly sensitive method for visualizing protein spots, with a detection limit in the low- to mid-nanogram level. But recent studies have indicated that it might chemically modify some proteins, resulting in poor yields of certain peptides. The technique also suffers from being chemically incompatible with the electroelution of proteins from a gel onto a membrane support such as nitrocellulose.

India ink, with a detection limit on a par with that of silver, offers an alternative method that has been used extensively to stain membrane-immobilized proteins. Because India ink staining is based on noncovalent interactions between proteins and colloidal carbon, it should not modify the proteins and thereby interfere with MS analysis. But Klaus Klarskov and Stephen Naylor, then at the Mayo Clinic (*www.mayo.edu*), wanted to test this theory (*1*).

The researchers, now at the University of Sherbrooke (*www.usherb.ca*) and Beyond Genomics (*www.beyondgenomics.com*), respectively, compared the mass spectra of proteins blotted onto nitrocellulose, stained with India ink, and digested, with those of proteins stained in-gel with silver or Coomassie blue and digested in-gel. They found that the India ink-treated proteins yielded the highest number of peptides. The researchers reasoned that this result might have been due to more complete proteolytic digestion on the membrane as compared to in the gel.

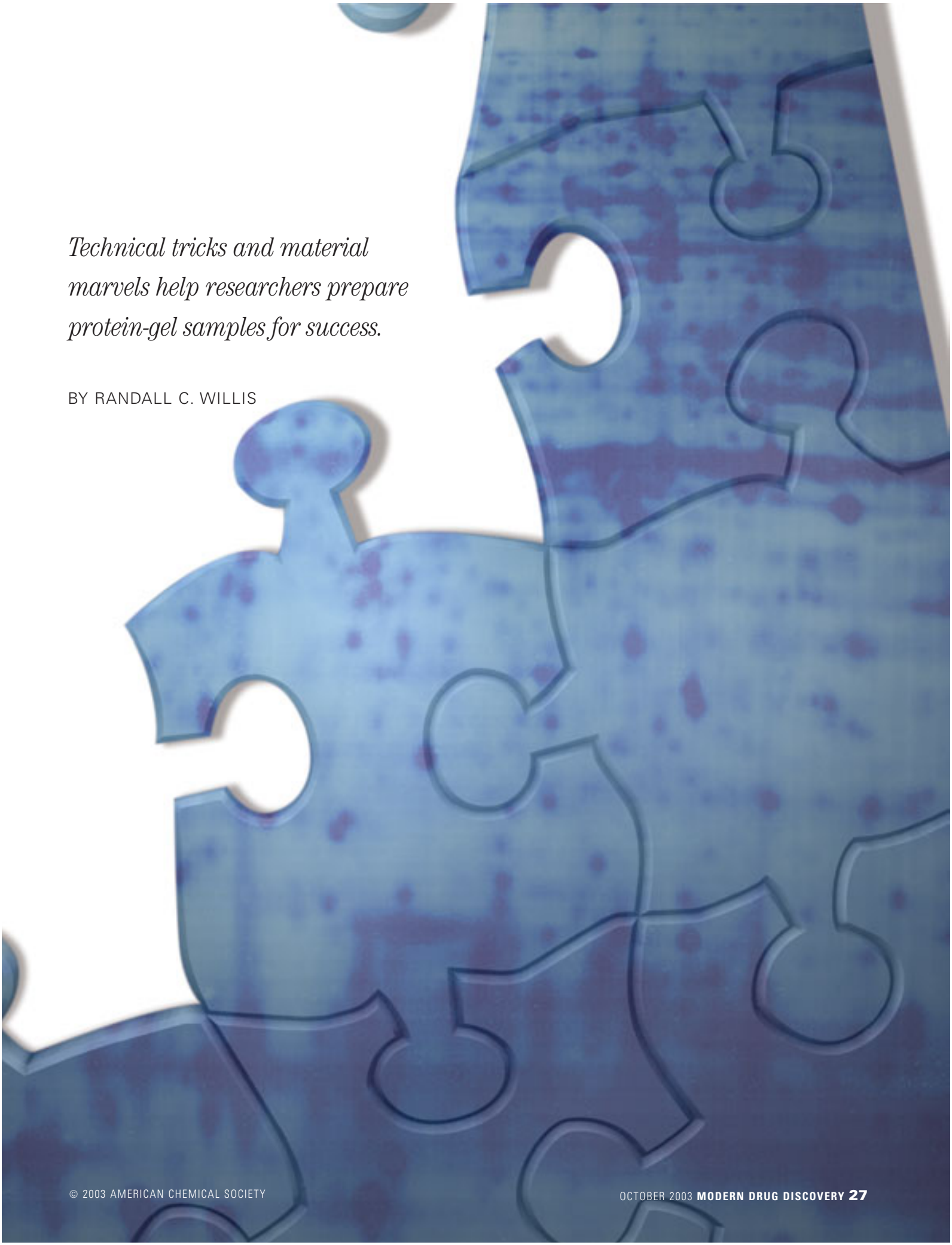
The researchers then tested the system in a "real world" setting, looking for proteins that were covalently modified during treatment with a nonsteroidal anti-inflammatory drug (NSAID). They isolated serum proteins from a drug-treated rat on a 2-D gel that they blotted and stained with India ink. The researchers then probed the blot with antibodies directed against the NSAID to see which proteins had bound the drug, and they noticed three modified protein spots, which they excised, digested, and analyzed by matrix-assisted laser desorption-ionization (MALDI) MS. They compared the resulting peptide sequences with those in the National Center for Biotechnology Information protein database (*www.ncbi.nlm.nih.gov*) and determined that the peptides mapped to the α-, β-, and γ-chains of fibrinogen. Why the drug modified these proteins, however, was unclear, but the two scientists believe that the sensitivity of the method makes it invaluable.

Looking for a needle

Like silver, India ink is a nonspecific stain, marking all of the proteins in a sample indiscriminately. When a researcher is looking for a global change in protein expression, these methods work well, but in many cases, the scientist already knows what kind of changes to look for. In this situation, staining all proteins results in a lot of noise and little signal of interest. To address this issue, several companies have developed stains that target specific protein modifications, such as phos-

Technical tricks and material marvels help researchers prepare protein-gel samples for success.

BY RANDALL C. WILLIS



phorylation or glycosylation. By having to identify only the proteins to which molecular changes have occurred, researchers save time and effort.

Wayne Patton and colleagues at the University of Oregon (*www.uoregon.edu*) and Molecular Probes, Inc. (*www.probes.com*), used a novel fluorescent dye (Figure 1) to probe the phosphorylation state of proteins in bovine heart cell mitochondria, organelles involved in cellular respiration and apoptosis-signaling pathways (*2*). The researchers isolated proteins from the mitochondrial extracts by 2-DE and stained the gels first with the Pro-Q Diamond stain to specifically indicate phosphorylated proteins and then with SYPRO Ruby stain to examine all proteins in the gel. They then generated differential display maps of the two staining techniques and compared the protein expression patterns using Z3 software (Compugen, *www.cgen.com*).

The researchers excised the phosphate-specific spots from the gel and performed peptide mapping using MALDI time-of-flight (TOF) MS to identify the phosphoproteins. Using this method, they were able to determine that three of the five isoforms of a member of a protein complex were phosphorylated to varying degrees.

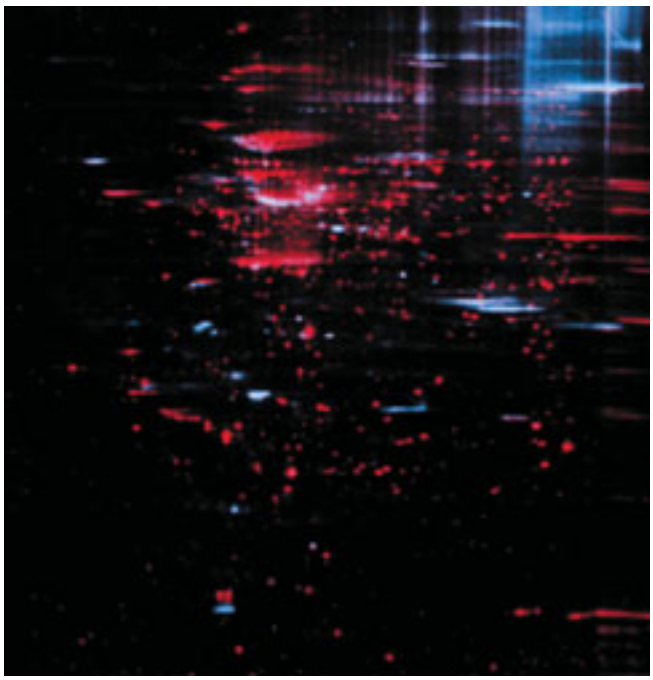


Figure 1. Looking for needles. By using a stain specific to phosphorylated proteins, researchers can quickly locate phosphoprotein needles (blue) in a total protein (red) haystack. (Image courtesy of Molecular Probes, Inc.)

Cutting to the quick

As stated earlier, proteomic analysis typically involves the cleavage of proteins with a protease followed by MS analysis. But during sample workup, most proteins exist at low (submicromolar) concentrations in large volumes (hundreds of microliters), and the commonly used endopeptidases have Michaelis constant (K_m) values of 5–50 mM, which means that protein cleavage is inefficient. Simply raising the protease concentration, however, leads to enzyme self-cleavage, which can swamp the MS signal, and protein preconcentration is often not amenable to automation.

To address these problems, several research groups and companies have developed methods for the digestion of proteins by enzymes immobilized on some form of support. Early efforts involved researchers using enzymes immobilized on resin beads, such as POROSzyme beads from PerSeptive Biosystems (now Applied Biosystems, *www.appliedbiosystems.com*). Similar products are also available from companies like Pierce Biotechnology (*www.piercenet.com*).

More recently, however, researchers have bound the enzymes directly to the walls of microfluidic chambers or capillaries (*3*). A potential problem with the microfluidic or capillary-based systems, however, is the small amount of enzyme that can be bound. And because the surface-to-volume ratio of these systems is small, the effective activity of the enzyme is often reduced.

To address these problems, Jean Frechet and colleagues at the Lawrence Berkeley National Laboratory (*www.lbnl.gov*) and the University of California, Berkeley (*www.berkeley.edu*), developed a capillary microreactor that contains a porous polymer monolith to increase the effective surface area of the capillary and trypsin immobilized at the end of azlactone chains (*4*). Using a robotic interface, the researchers injected a series of proteins into their microreactor and spotted the resulting samples onto a MALDI plate. They then performed MALDI MS on the samples to determine how well the proteins digested. Although they never achieved 100% cleavage, the researchers thought that their results compared well with earlier microfluidic attempts.

MS microprep

Achieving microscale concentration and purification of proteolysis-generated peptides for MS analysis has generally run into some challenges involving retention by reversed-phase beads in the microcolumn, ease and reproducibility of column packing, robustness of the column, and recovery of very small amounts of peptide material. To improve this protein digestion/MS interface, Matthias Mann and colleagues from the University of Southern Denmark (Odense) designed a novel microtip procedure, using what they call Stop and Go Extraction tips, or StageTips, for sample pretreatment (Figure 2, *5*).

Central to the StageTip design is a Teflon matrix embedded with C18 beads formed into a small (~60-nL) disk-shaped membrane. The Teflon material and minute disk volume discourage irreversible peptide absorption. The fixed position of the beads—as opposed to loose packing material used in traditional solid-phase extraction—enables a large diameter and short column length and facilitates the equal flow of analyte solution throughout the entire disk.

Moreover, the membrane can be held in place solely by the perimeter of a vessel such as a pipet tip, without the need for additional support. This further minimizes flow irregularities and simplifies StageTip assembly. Fabrication simply involves corking out the disks from a commercially available C18 bead-embedded Teflon mesh and placing them into pipet tips. An occasional user can easily manufacture about five of these tips per minute, say the scientists, with a materials cost of less than $0.01/disk.

The researchers evaluated the C18 StageTips with stable-isotope-labeled digests. They used bovine serum albumin (BSA) digested

with trypsin and prepared a dilution series of these digests to determine the usefulness of StageTips for low sample amounts, finding that they could achieve complete sample recovery down to peptide concentrations of 0.5 fmol/µL. On the opposite end of the spectrum, the researchers judged the loading capacity of the device using a combination of measurements on the BSA digests and on digests of the soluble lysate from *E. coli*, determining a capacity of 2–4 µg of peptide per 0.4 × 0.5 mm disk—about 50 pmol of protein—which is larger than most proteomic MS analyses requirements. By simply stacking additional disks, the column length can be increased for greater capacity and the extraction of more-complex samples.

Of course, few researchers have the desire or engineering ability to design their own isolation tips. Thus, several companies have jumped into the fray by providing researchers with similar tools. For example, at the recent meeting of the American Society for Mass Spectrometry (*www.asms.org*) in Montreal, researchers from Advion Biosciences (*www.advion.com*) introduced the E-Tip Sample Cleanup Option for use with their electrospray ionization (ESI) MS platform. Based on the ZipTip technology developed by Millipore (*www.millipore.com*), which offers a variety of resin types including C18, cation exchange, and metal chelate, the E-Tip allows users to rapidly desalt, purify, and concentrate microliter-scale biological samples. At the same meeting, officials from Genomic Solutions (*www.genomicsolutions.com*) announced the release of their own PrepTip technology.

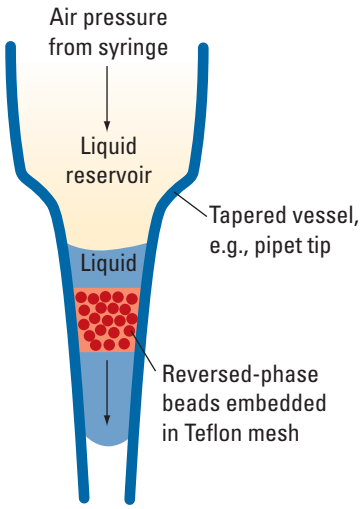


Figure 2. Preparation tips. (Adapted with permission from Ref. 5.)

Extracting MALDI results

Once the sample has been prepared, it still has to be run in the spectrometer. With ESI MS, liquid samples can be injected directly into the unit, but with MALDI MS, the sample must first be mixed with matrix and deposited on a metal plate. The oldest technique for sample deposition is the dried-droplet method, which involves placing sample–matrix mixtures on the plate and then allowing the solvent to evaporate. Although this method has been refined extensively over the past decade into a robust technique, it is not without its shortcomings.

Because the method produces heterogeneous matrix–analyte deposits, researchers might need to move the laser across the sample to find analyte-rich positions. Furthermore, when used for low-femtomole detection studies, the method suffers from reduced ion yields and poor detection efficiency of post-translationally modified peptides. To address these limitations, Sven Kjellström and Ole Nørregaard Jensen of the University of Southern Denmark (*www.sdu.dk/Nat/bmb*) recently developed a liquid–liquid extraction (LLE) system for partitioning hydrophobic and hydrophilic peptides in situ on the MALDI plate (*6*).

To perform LLE, the researchers place a droplet of peptides in aqueous buffer onto the MALDI plate and then add a second droplet that contains matrix in water-immiscible organic solvent. The hydrophobic peptides move from the aqueous to the organic phase, and the efficiency of this movement is affected by factors such as amino acid sequence, pH and ionic strength of the aqueous phase, and the nature of the solvent. Once the organic-matrix phase dries and crystallizes, the scientists move the remaining aqueous material to a new spot on the MALDI plate, where the process is either terminated with the addition of matrix or repeated.

The researchers initially tested their system with a mixture of 3- to 30-kDa proteins, finding that the hydrophobic proteins extracted more efficiently into organic solvent plus matrix than into organic solvent alone. Also, multiple rounds of LLE were often required for best results. They then performed LLE on a tryptic digest of phosphorylated β-casein and found that the more-hydrophobic peptides partitioned into the organic phase, whereas the phosphopeptides remained and were effectively concentrated in the aqueous phase.

The researchers then examined whether LLE would effectively partition other post-translationally modified peptides by performing extraction on a tryptic digest of BSA spiked with a phosphopeptide, glycopeptide, and acetylated peptide. As they had hoped, they detected the phosphopeptide and glycopeptide in the aqueous phase and the acetylated peptide in the organic phase. The scientists also determined that after an extensive washing procedure prior to in-gel digestions, LLE was effective for the MALDI MS analysis of protein bands from SDS-PAGE and 2-DE. Future development of the LLE method is aimed at expanding the technique for the analysis of other hydrophobic biomolecules, including membrane proteins.

The bottom line

While there is many a slip betwixt cup and lip—or in this case, between gel and spectrometer—corporate and academic researchers are making every effort to minimize the effects of these potential proteomic hazards.

References

(1) Klarskov, K.; Naylor, S. *Rapid Comm. Mass Spectrom.* **2002,** *16,* 35–42.
(2) Schulenberg, B.; et al. *J. Biol. Chem.* **2003,** *278,* 27251–27255.
(3) Mao, H.; Yang, T.; Cremer, P. S. *Anal. Chem.* **2002,** *74,* 379–385.
(4) Peterson, D. S.; et al. *J. Proteome Res.* **2002,** *1,* 563–568.
(5) Rappsilber, J.; Ishihama, Y.; Mann, M. *Anal. Chem.* **2003,** *75,* 663–670.
(6) Kjellström, S.; Jensen, N. *Anal. Chem.* **2003,** *75,* 2362–2369.

Randall C. Willis is a senior associate editor of *Modern Drug Discovery*. Send your comments or questions about this article to mdd@acs.org or to the Editorial Office address on page 3. ■

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