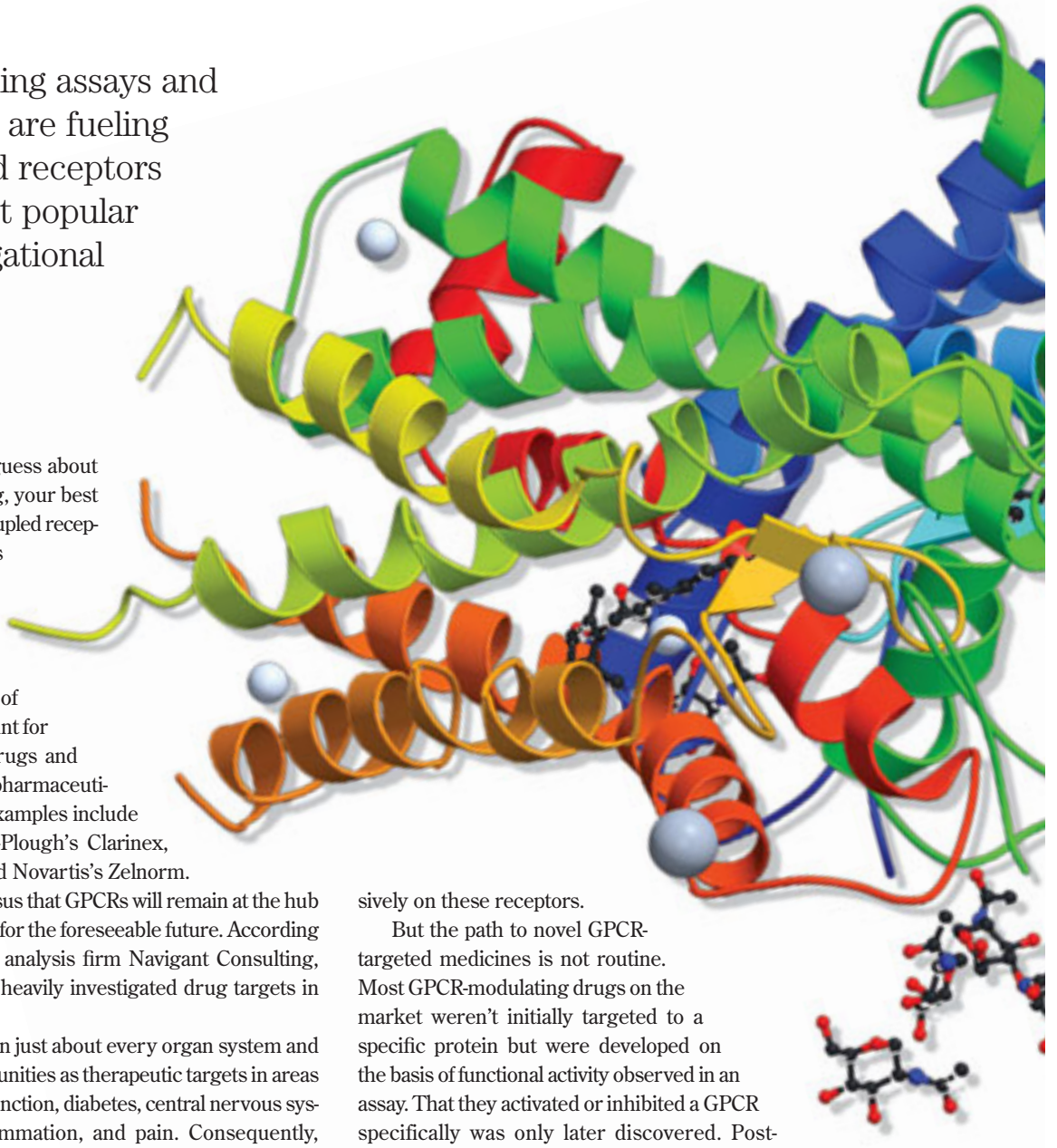
It's a GPC

Cell-based screening assays and structural studies are fueling G-protein coupled receptors as one of the most popular classes of investigational drug targets.

BY DAVID FILMORE



RHODOPSIN IMAGE: PROTEIN DATA BANK

If you had to make a wild guess about the target of a certain drug, your best odds are with "G-protein coupled receptor." Drugs targeting members of this integral membrane protein superfamily, which transmit chemical signals into a wide array of different cell types, represent the core of modern medicine. They account for the majority of best-selling drugs and about 40% of all prescription pharmaceuticals on the market. Notable examples include Eli Lilly's Zyprexa, Schering-Plough's Clarinex, GlaxoSmithKline's Zantac, and Novartis's Zelnorm.

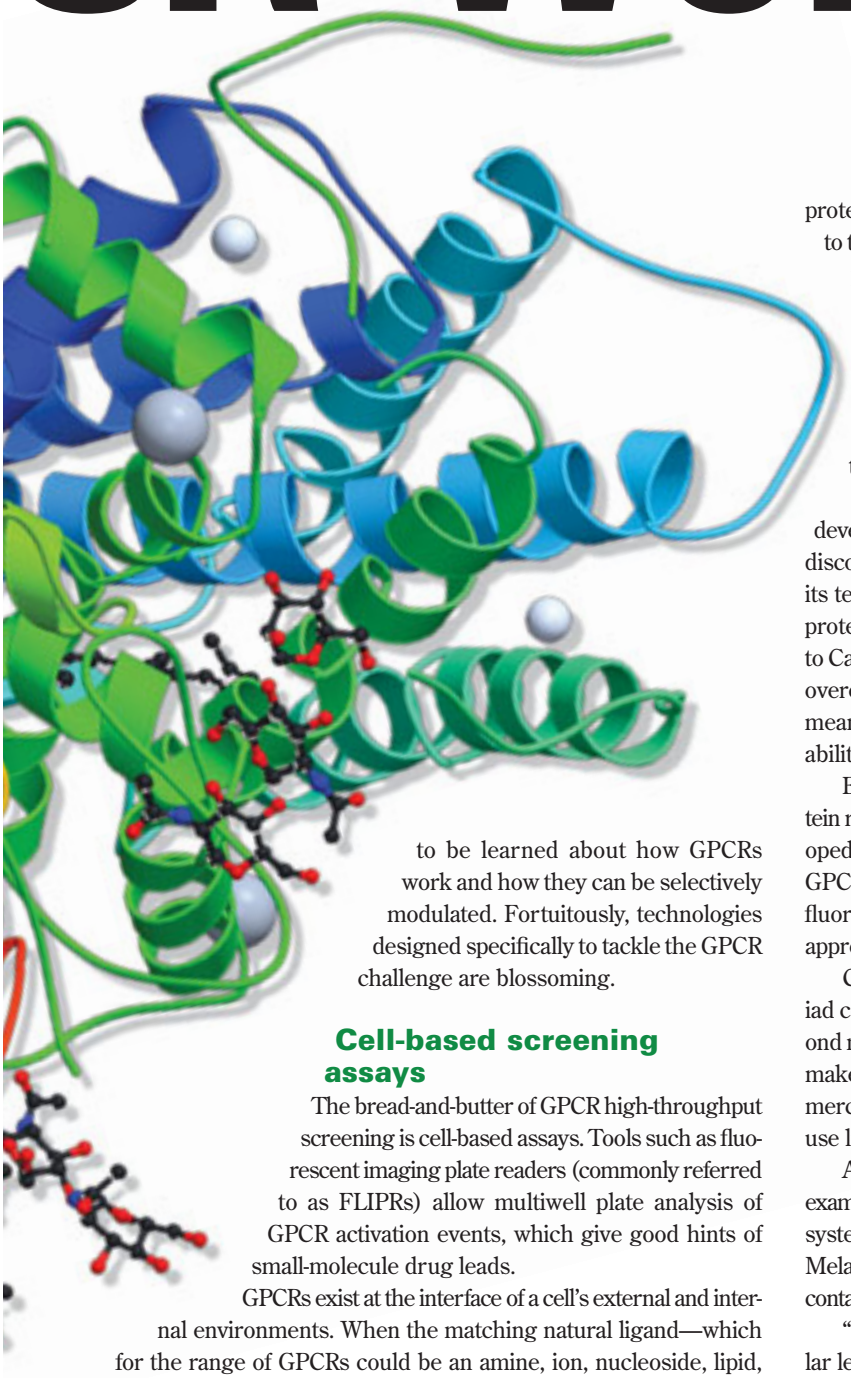
And there is broad consensus that GPCRs will remain at the hub of drug development activities for the foreseeable future. According to a recent report by market analysis firm Navigant Consulting, "GPCRs are among the most heavily investigated drug targets in the pharmaceutical industry."

These proteins are active in just about every organ system and present a wide range of opportunities as therapeutic targets in areas including cancer, cardiac dysfunction, diabetes, central nervous system disorders, obesity, inflammation, and pain. Consequently, GPCRs are prominent components of pipelines in small and large drug companies alike, and many drug discovery firms focus exclusively on these receptors.

But the path to novel GPCR-targeted medicines is not routine. Most GPCR-modulating drugs on the market weren't initially targeted to a specific protein but were developed on the basis of functional activity observed in an assay. That they activated or inhibited a GPCR specifically was only later discovered. Post-Human Genome Project, however, targets are the starting points for most drug discovery endeavors. And there is still much

© 2004 AMERICAN CHEMICAL SOCIETY

CR world



to be learned about how GPCRs work and how they can be selectively modulated. Fortuitously, technologies designed specifically to tackle the GPCR challenge are blossoming.

Cell-based screening assays

The bread-and-butter of GPCR high-throughput screening is cell-based assays. Tools such as fluorescent imaging plate readers (commonly referred to as FLIPRs) allow multiwell plate analysis of GPCR activation events, which give good hints of small-molecule drug leads.

GPCRs exist at the interface of a cell's external and internal environments. When the matching natural ligand—which for the range of GPCRs could be an amine, ion, nucleoside, lipid, peptide, protein, or, for optical receptors, light—comes along, it binds to a receptor's active site and causes a conformational change in the protein to form its active state. This signals the G protein coupled to the receptor inside the cell to release components that set some predefined cellular mechanism in motion.

The trick for high-throughput cellular screening is to find a robust marker to monitor in cells overexpressing the GPCR of interest.

Calcium ions are one popular choice. Ca^{2+} is naturally produced in cells upon activation of GPCRs coupled to G_q proteins—one of the three main families of G proteins.

The Brussels-based company Euroscreen, for instance, has developed the AequoScreen assay to fuel its own GPCR-based drug discovery programs—as well as those of companies that purchase its technology. AequoScreen is based on a jellyfish-derived photoprotein called aequorin, which displays photoactivity proportional to Ca^{2+} concentration. Screening a library against an array of GPCR-overexpressing cells mixed with aequorin provides a quantitative means of assessing a compound's ability to activate a GPCR (or its ability to antagonize activation).

Even though intracellular Ca^{2+} levels rise directly only from G_q-protein receptor activation, genetic expression methods have been developed that allow Ca^{2+} production to proceed upon activation of GPCRs coupled to other G protein types (i.e., G_i/G_o or G_s). Thus, fluorescent Ca^{2+} screening has become somewhat of a universal approach to screening small-molecule libraries against GPCRs.

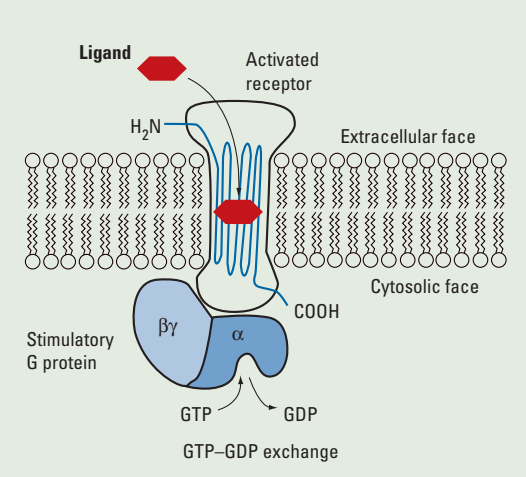
Cyclic adenosine monophosphate (cAMP), which controls myriad cellular metabolic pathways, is one of the most important "second messenger" compounds of the GPCR activation process. It also makes a good high-throughput screening marker. Numerous commercially available and individually made cell-based GPCR assays use luminescent tags that bind to cAMP.

Arena Pharmaceuticals' entire drug screening program, for example, is based around the cAMP approach, although the company's system doesn't require the use of tagging compounds. With its Melanophore technology, it expresses GPCR targets in frog skin cells containing a pigment that is highly sensitive to changing levels of cAMP.

"If you stimulate these cells such that they increase intracellular levels of cAMP inside the cell, the pigment disperses throughout the cell and appears black," explains Dominic Behan, co-founder and chief scientific officer of Arena Pharmaceuticals. "And if you

stimulate these cells to decrease the level of cAMP, the pigment aggregates to the center and the cell appears clear." Because G_s- and G_q-coupled receptor activation stimulates cAMP production, whereas G_i/G_o-coupled receptors inhibit cAMP, this is a broadly applicable screening assay.

A more straightforward approach to a universal GPCR assay is to monitor a mechanism common and consistent for all receptor–G-protein couplings. GPCR desensitization following ligand activation is, perhaps, the only process identified that fits this requirement. The need to continually increase dosages of morphine, which targets the GPCR μ-opiate receptor, to maintain a constant clinical effect is a familiar example of this desensitization process.



Signal send-off. Ligand binding to a GPCR's extracellular region triggers changes in the protein's transmembrane region. This causes the release of guanosine diphosphate (GDP) and the uptake of guanosine triphosphate (GTP) from the G protein, spurring activation of predefined signaling pathways.

"A receptor has to have the ability to signal independent of other GPCRs," says Carson Loomis, senior vice president for research at Norak Biosciences, a company focused exclusively on GPCR drug discovery and technology licensing. "That is why the G-protein signaling pathway is so diverse. But desensitizing has one common mechanism for virtually all [GPCRs]."

The desensitization pathway begins with binding of the cytoplasmic protein β-arrestin to the activated receptor, which "turns off" the GPCR. The receptor–arrestin complex then enters the cell, where the ligand is removed and the receptor is recycled back to the membrane. Norak's Transfluor assay is designed to monitor the highly characteristic movements of β-arrestin—into successive spatial arrangements called "pits" and "vesicles"—during this recycling process. By genetically engineering cell lines to express green fluorescent protein-tagged arrestin and to overexpress the GPCR of interest, various commercial imaging instruments can be used to measure and quantify GPCR activation.

Several other companies including 7TM, another GPCR-focused drug company, and Perkin-Elmer have developed GPCR screening technologies that take advantage of the arrestin–receptor binding. Each of these companies applies a technique called bioluminescence resonance energy transfer, which measures changes in light emission based on the interaction between an electron donor attached to β-arrestin and an acceptor expressed with the GPCR target.

Orphan attachments

Besides the many GPCRs that function as basic receptors for sensory functions like seeing and smelling, which are not prime therapeutic targets, there are more than 300 other GPCRs up for grabs for drug discovery initiatives. About 200 of these—a portion of which account for currently marketed GPCR drug targets—have known natural ligands. And ligands are not known for about 150 nonsensory receptors identified as GPCRs from the Human Genome Project. These so-called orphan GPCRs have become a primary focus of many investigators and companies, because of the largely uncharted path of discovery they offer.

Typically, an initial goal is to "deorphanize" these GPCRs using high-throughput screening. Determining the endogenous ligand provides a first hint of function, structural cues for lead design, and a particular receptor-activating entity to antagonize.

For example, in 1999, researchers at SmithKline Beecham (now GlaxoSmithKline) identified the orphan GPCR GPR14's natural ligand as urotensin II (UII), a cyclic peptide associated with cardiovascular homeostasis and pathology (*1*). More recently, researchers at Aventis found a potent nonpeptide GPR14/UII antagonist by screening UII analogues against GPR14-transfected cells in a Ca^{2+} FLIPR assay and designing small molecules based on the important pharmacophores for activating the receptor (*2*).

Despite this type of success, many scientists focused on discovering new drugs appear to be bypassing the conventional deorphanizing step.

"It is really hard to develop peptide libraries to look for the ligand," Loomis says. Instead, he notes, drug researchers more often perform initial high-throughput assays to find synthetic small-molecule agonists, which then can be used to "go back to the cell and work out the physiology of the receptor," he adds.

Arena Pharmaceuticals, on the other hand, does substantial reconnaissance work on potential GPCR targets—specifically using genomic analysis for determining expression in specific organs and cells of interest and the intracellular signaling mechanism—prior to having any knowledge of natural or nonnatural ligands. The company subsequently uses its constitutively activated receptor technology (CART), a generic technique to "trick GPCRs into activation," Behan says, by mutating key sequence regions. CART allows researchers to screen libraries against an orphan's active state without the need for a ligand.

By forcing GPCR signaling, scientists can readily validate agonist response and directly determine antagonists and inverse agonists (i.e., ligands that expressly deactivate targets).

"If you wait too long to identify the natural ligand, you will miss out on the opportunity of finding the actual drugs," Behan explains.

Structurally speaking

Assays like those designed by Norak, 7TM, and Arena are helping to populate pipelines. But there is some consensus that more structural data is needed to truly exploit the value of GPCRs.

"It is absolutely imperative" to have more structural information, Loomis says. "These receptors have so many subtypes," he explains. "[For example,] I think there are 16 serotonin receptors. The importance of being able to target one and not the others is critical. You can't get that kind of specificity in compounds that are already binding at 10^{-9} M without some structural work."

"But right now," he adds, "it is trial and error."

GPCRs, like other membrane proteins, are notoriously difficult to crystallize. All GPCRs are known to have a common motif of seven transmembrane helical structures, but the only GPCR crystal structure published at atomic resolution is of the inactive conformation of rhodopsin, the optical receptor protein solved in 2000 by University of Washington chemistry and ophthalmology professor Krzysztof Palczewski (*3*). Although GPCR crystallography is the focus of several companies' business plans, little else has been accomplished since this report.

"Orphan" GPCRs have become a primary focus of many investigators and companies, because of the largely uncharted path of discovery they offer.

"The key bottleneck of GPCR crystallography is sample," says Juan Ballesteros, CSO of Novasite, a GPCR-focused drug discovery company that retains Palczewski as a crystallography consultant. Obtaining high-quantity and high-purity GPCR proteins is very challenging, because membrane proteins are typically produced in a heterogeneous manner by cells with substantial variability in glycosylation, Ballesteros explains.

But Novasite has developed a new approach using retinas from transgenic frogs and mice as GPCR bioreactors. It takes advantage of the efficient mechanism by which the eye produces rhodopsin to express other GPCRs. "Every second, you are expressing 80,000 new rhodopsin molecules that are 98% chemically identical," Ballesteros says.

Novasite will soon publish a structure of rhodopsin in its active state, which, he says, will itself be a significant accomplishment and will also validate the new expression system. The company then plans to use this expression system to generate receptor samples for 20 GPCR therapeutic targets and start crystallization trials by the end of 2004.

This will have "tremendous value to the drug discovery effort," Ballesteros insists. Nevertheless, with more than 300 potential GPCR targets of interest, crystallization efforts clearly have a long way to go.

Receptor structures are sometimes able to inform drug discovery efforts through extrapolation from rhodopsin's structure. Researchers from the University of Michigan and the University of Kansas, for example, recently used computational homology modeling techniques to determine a three-dimensional structure of dopamine 3 (D_3), a potential target for drug addiction, Parkinson's disease, and schizophrenia (*4*). They found potential ligands via computational pharmacophore and structure-based screening, several of which displayed substantial inhibition in a D_3 binding assay.

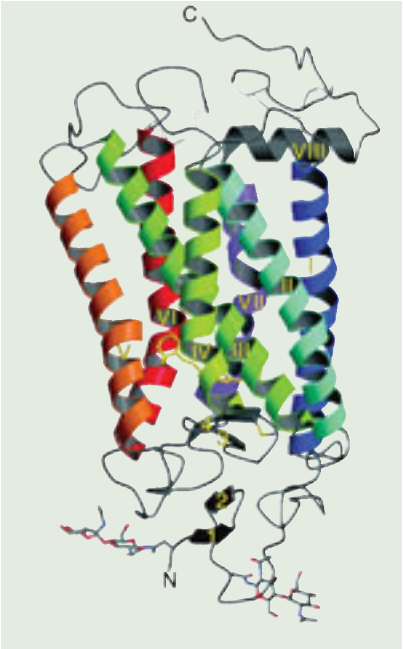
Ballesteros notes that the rhodopsin structure can serve as a useful guide for family 1 GPCRs, which are homologous to the optical protein, but it provides less practical information for the other two GPCR families. "The farther away [structurally] from rhodopsin, the more valuable" is a target GPCR structure, he stresses.

Predix Pharmaceuticals, however, has developed an in silico GPCR structure-based method that does not rely on rhodopsin homology. Its PREDICT algorithm combines protein sequence information with membrane environment property factors to determine the most stable three-dimensional structure of a receptor's transmembrane domain. The company recently published five examples of successful early-stage discovery projects that led to "very promising lead compounds" validated via in vitro and in vivo assays (*5*). Each was initiated by screening libraries virtually against PREDICT-generated structures, including of two different serotonin receptors.

However, according to Ballesteros, "the new hot thing" is allosteric GPCR modulators. These are compounds that bind away from a protein's active site and modulate its activity independent of the natural ligand. "Big pharmaceutical companies are now very interested in this," he says. Amgen's Sensipar, approved by the FDA in March and indicated for secondary hyperparathyroidism and elevated calcium levels, became the first allosteric GPCR modulator. There are others in several different company pipelines.

Computational tools are not often very effective at modeling allosteric binding sites, he says. "This is where you really need the structure of a particular receptor to guide discovery," he stresses.

Another tool for structure-based development, whether in the active site or in an allosteric region, is high-throughput mutagenesis screening, in which different mutations at a GPCR binding site are analyzed against multiple ligands. This helps uncover the key ligand–receptor contacts responsible for drug recognition by the receptor. By this means, the information unearthed in functional assays is connected to structural data determined by X-ray crystallography or computation.



Visual clue. The visual photoreceptor rhodopsin, shown above in its inactive state, is the only GPCR with a solved crystal structure. (Adapted with permission from Ref. 3.)

Place your bets

Norak, Arena, 7TM, Novasite, and Predix are prime examples of firms completely focused on the GPCR drug discovery effort. And the extensive partnerships and licensing agreements each has with the likes of Aventis, Merck, Eli Lilly, AstraZeneca, Hoffman-La Roche, and individual endeavors by other big pharmaceutical companies point to the far-reaching investments ongoing in this area. Rather than a wild guess, GPCRs seem to be a good bet for future drug discovery successes.

References

(1) Ames, R. S.; et al. *Nature* **1999,** *410,* 282–286.
(2) Flohr, S.; et al. *J. Med. Chem.* **2002,** *45,* 1799–1805.
(3) Palczewski, K.; et al. *Science* **2000,** *289,* 733–734.
(4) Varady, J.; et al. *J. Med. Chem.* **2003,** *46,* 4377–4392.
(5) Becker, O. M.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **2004,** *101,* 11304–11309. ■