Drug discovery in academia

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Verkman, A. S. Drug discovery in academia. Am J Physiol Cell Physiol 286:
C465–C474, 2004;10.1152/ajpcell.00397.2003.—Drug discovery and development
is generally done in the commercial rather than the academic realm. Drug discovery
involves target discovery and validation, lead identification by high-throughput
screening, and lead optimization by medicinal chemistry. Follow-up preclinical
evaluation includes analysis in animal models of compound efficacy and pharma-
cology (ADME: administration, distribution, metabolism, elimination) and studies
of toxicology, specificity, and drug interactions. Notwithstanding the high-cost,
labor-intensive, and non-hypothesis-driven aspects of drug discovery, the academic
setting has a unique and expanding niche in this important area of investigation. For
example, academic drug discovery can focus on targets of limited commercial
value, such as third-world and rare diseases, and on the development of research
reagents such as high-affinity inhibitors for pharmacological “gene knockout” in
animal models (“chemical genetics”). This review describes the practical aspects of
the preclinical drug discovery process for academic investigators. The discovery of
small molecule inhibitors and activators of the cystic fibrosis transmembrane
conductance regulator is presented as an example of an academic drug discovery
program that has yielded new compounds for physiology research and clinical
development.

high-throughput screening; drug development; pharmacology; fluorescence; cystic
fibrosis transmembrane conductance regulator

Drug discovery is the process whereby compounds with activity against a specified target or function are identified, evaluated, and optimized for clinical applications. As diagrammed in Fig. 1, the initial step in drug discovery involves the identification and validation of a target, generally a gene product, for which pharmacological modulation of target activity is predicted to produce a desired effect. Examples are inhibition of a gene product responsible for early brain swelling after stroke or activation of the defective product of a mutant gene causing a genetic disease. Typical goals include enzyme inhibitors, receptor agonists or antagonists, and transporter inhibitors or activators. Target identification and validation may involve gene/protein expression profiling, phenotype analysis in cell culture and transgenic mouse models, and evaluation of humans with gene deletion/mutation (21). The initial identification of candidate “lead” compounds usually involves high-throughput screening (HTS; see Table 1 for common abbreviations used in drug discovery) of diverse small molecule collections or structurally selected compounds with known or theoretically predicted activity against a target (8). Initial drug discovery thus requires a robust assay of target activity and a collection of compounds for testing. “Hits” from initial screening are evaluated on the basis of many criteria, including but not limited to potency, specificity, toxicity, pharmacology, biopharmaceutical properties, and efficacy in animal models, to select lead compound(s) for optimization by synthetic chemistry and more extensive preclinical evaluation in animal models (4, 20). These preclinical data form the basis of the preclinical pharmacology section of an investigational new drug (IND) application to carry out clinical trials (25).

Drug discovery and development is generally done in the commercial realm because of its high cost, the requirement for personnel with multidisciplinary expertise, and the intrinsic high-risk/high-reward and non-hypothesis-driven nature of the work. The high-cost and non-hypothesis-driven aspects of drug discovery present challenges in funding in an academic setting; however, as discussed below (see Table 2), the costs are comparable to those of typical institutional core facilities. Another challenge in carrying out non-hypothesis-driven research in an academic setting is attracting competent scientific and technical personnel, particularly for the screening, compound optimization, and animal testing aspects of the research. However, the prospects of applying new compounds to interesting scientific problems are attractive to junior investigators, as are the training and commercial aspects of the work. For example, inhibitors of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel identified by a drug discovery program have been useful in testing the role of CFTR in submucosal fluid secretion in excised human airways (38), a problem that has been difficult to address using chronically diseased airways from cystic fibrosis subjects (18). Finally, the high-risk/high-reward aspect of drug discovery is more of an issue in the commercial realm for the approval and marketability of new compounds. Fewer than one of four compounds that enter clinical testing is ultimately approved for marketing, with some reasons for failure including poor pharmacology, unexpected toxicity in humans, failure to achieve efficacy in clinical trials, and the emergence of better leads. The average preapproval development cost has been estimated.
to be as high as US$800 million per compound (7), with a 7-year average time from initial IND filing until approval (31). Clinical trials make up the majority of the costs. The preclinical costs from lead identification to IND filing, usually taking 1–3 years, is typically US$2–4 million. The number of new targets has been decreasing over the last few years, as has the number of new chemical entities (NCEs) entering the drug development pipeline from major pharmaceutical companies (<2 NCEs per major drug company) (9).

It has been argued that drug discovery in an academic setting is urgently needed to explore alternative paradigms in the currently very inefficient drug discovery process. At present, realistic goals for an academic drug discovery program can include, for example, the identification of reasonably potent and specific reagents for protein inhibition/activation in animal models and the discovery and evaluation of drug candidates with the intention of partnering with industry for further development. Although there is wide variability, approximately 1 of 2,000–5,000 random small molecules has significant (though maybe weak) inhibitory activity against a target function, making the identification of useful research reagents a quite realistic goal in an academic drug discovery program.

This review focuses on the scientific and practical aspects of the preclinical drug discovery process from an academic perspective. Issues in assay development, compound screening, evaluation, and optimization are presented. This review does not deal with clinical trials, intellectual property issues, chemical, manufacturing, and control (CMC) issues, regulatory considerations, or commercialization. The identification of CFTR inhibitors and activators is discussed as an example of drug discovery in an academic setting.

**THE DRUG DISCOVERY PROCESS**

Assay development for compound screening. Development and selection of a screening assay is arguably the most important aspect of the drug discovery process. As a surrogate for clinical efficacy, the screening assay should be designed to assay target activity with high sensitivity and specificity in a setting that mimics where possible the in vivo target dynamics. The identification of inhibitors of a purified enzyme having a unique function and giving a colorimetric readout is much more simple conceptually and more simple to implement than a complex cell-based assay involving sequential targets. For example, an important goal of cystic fibrosis drug discovery is the identification of compounds that correct defective intracellular processing of the ΔF508-CFTR mutant protein, whose gene is present in at least one allele in ~90% of subjects with cystic fibrosis. ΔF508-CFTR is retained at the endoplasmic reticulum in epithelial cells and has an intrinsic defect in its chloride channel gating function. An assay of functional correction of ΔF508-CFTR requires consideration of the cell biology of protein folding and defective channel gating in the

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**Table 1. Glossary of drug discovery terms**

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADMET</td>
<td>Administration, distribution, metabolism, elimination, toxicology</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve in pharmacokinetics</td>
</tr>
<tr>
<td>DDI</td>
<td>Drug-drug interactions</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
</tr>
<tr>
<td>IND</td>
<td>Investigational new drug (application)</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamics</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>TOX</td>
<td>Toxicity analysis</td>
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**Table 2. Costs of a drug discovery program**

<table>
<thead>
<tr>
<th>Instrumentation</th>
<th>Cost</th>
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</thead>
<tbody>
<tr>
<td>Robotic high-throughput screening apparatus</td>
<td>&gt;$500,000</td>
</tr>
<tr>
<td>Annual maintenance fees: 5–10% of initial cost</td>
<td></td>
</tr>
<tr>
<td>Informatics</td>
<td>Server hardware, programming: $150,000</td>
</tr>
<tr>
<td>Annual maintenance fees: 5–15% of initial cost</td>
<td></td>
</tr>
<tr>
<td>Compounds for screening</td>
<td>Compound purchase (0.1–0.5 mg): $1–2 per compound</td>
</tr>
<tr>
<td>Disposable supplies for screening</td>
<td>$0.20–10 per well screened</td>
</tr>
<tr>
<td>Compounds for hit evaluation and lead optimization</td>
<td>Synthesis hardware: &gt;$500,000</td>
</tr>
<tr>
<td>Annual maintenance fees: 5–10% of initial cost</td>
<td></td>
</tr>
<tr>
<td>Synthesis supplies: $50–150 per compound to produce 50-mg quantities</td>
<td></td>
</tr>
<tr>
<td>Personnel</td>
<td>Cell culture (1 full-time equivalent per project)</td>
</tr>
<tr>
<td>Screening (1–2)</td>
<td></td>
</tr>
<tr>
<td>Database management (1)</td>
<td></td>
</tr>
<tr>
<td>Synthetic/medicinal chemistry (2–5)</td>
<td></td>
</tr>
<tr>
<td>Animal testing/pharmacology (&gt;2)</td>
<td></td>
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</tbody>
</table>
appropriate target cell so that the dynamics of ΔF508-CFTR activation in a nonepithelial cell type might have little relevance to human epithelial cells in vivo. Great care is thus required in assay design because of the substantial investment in resources for follow-up evaluation of active compounds identified in a primary screen. Similar contextual difficulties arise in most major classes of drug targets, with notable examples including G protein-coupled receptors, ion channels, and nuclear hormone receptors. Often logistical issues will force the use of a less relevant assay as a primary screen, such as isolated protein function, with more informative phenotypic assays done during secondary evaluation.

The requirements of a screening assay include good target sensitivity and specificity, robust readout, day-to-day reproducibility, technical simplicity, suitability for automation, and low cost. The technical and cost aspects of a screening assay are particularly important if the assay is to be repeated on hundreds of thousands of test compounds by utilizing HTS technology. The total per-assay cost of a single-point optical readout of activity in a cell-free system can be as low as US$0.10–0.50, whereas the cost of a single, complex cell-based assay can exceed US$5.00–10.00. A complex cell-based assay may require multiple reagent additions and washings over many hours or days. Per-assay costs include cell culture, solutions and reagents, and disposable items such as multiwell assay plates. Related operating expenses include instrumentation and compound costs as well as support for personnel involved in cell culture, assay setup, instrument operation, and data management. Assays that are amenable to optical readouts (absorbance, fluorescence, luminescence) are usually more robust, easily automated, and less expensive than assays utilizing radioisotopes or electrophysiology.

**High-throughput screening.** The implementation of a screening assay requires automation because of the repetitive and labor-intensive nature of compound screening. Several types of robotic instruments for automated compound screening are available commercially. An instrument set up by our research group is shown in Fig. 2. A robotic arm with 3-meter transit transports multiwell plates and pipette tips between workstations. Multiwell plates containing cultured cells are loaded onto a carousel in a CO₂ incubator having a rear entrance for robot access. A second carousel on the table surface is loaded with containers containing pipette tips and multiwell plates containing test compounds. The workstations shown in the photograph include a bar code reader, delidding station (to remove plastic lids of multiwell plates), cell wash station, constant-temperature incubator, plate shaker, liquid handling workstation, and two optical plate readers (absorbance, fluorescence, luminescence), each equipped with dual syringe pumps for solution additions during the assay. The liquid handling workstation accesses pipetting tools to add, remove, or transfer compounds/solutions from well to well or plate to plate. Other workstations that are integrated into the system as needed include a plate sealer (places a foil seal on multiwell plates containing test compounds), filtration workstation, and multiwell scintillation detector. All instrument operations and data collection are coordinated by a central computer. The instrument is programmed to carry out complex, interleaved operations over many hours or days without being monitored. In the event of instrument failure, which has occurred in <5% of 12- to 18-h procedures, the user is notified by beeper or cell phone to effect corrective action on-site or, if possible, off-site with the use of an Internet interface/web cam. Reasons for system failure have included power/vacuum interruption, plate mishandling during transfer operations, and failure of an individual workstation. Uninterrupted power supplies and strict system set-up procedures reduce these system failures. The initial cost of screening instrumentation is >US$500,000 with a typical annual maintenance fee of 5–10% of initial cost.

Screening assays are generally done by using standard 96- or 384-well plastic plates, although cell-based assays can be challenging in a 384-well format because of difficulties in cell plating, solution mixing, and washing procedures. Typically, 1,000–10,000 individual assays are carried out daily in HTS. Advances have been made recently to increase assay throughput (“ultrahigh throughput”) utilizing 1,536- or 3,456-well plates (<2 μl solution per well) and parallel multiwell camera-based optical readout. A recent trend is the use of high-throughput microscopy in the 96-well format coupled with automated image analysis. The term “high-content screening” has been coined to describe the analysis of multiple or complex parameters in screening assays, such as the intracellular distribution of fluorescence in single-cell image readouts (8). Also, various different paradigms have been introduced for HTS involving microfluidics, compound “chips,” and target microarrays (3, 16).

Data integrity and processing capability are important to the screening process. Multi-time-point assays can produce millions of readout points per day, and high-content screening with image readout can produce terabytes of data. Commercial bioinformatics software is available to capture information in relational databases for hypothesis testing and compound optimization. Various statistical parameters have been applied to evaluate assay sensitivity and specificity. A widely used statistical parameter is the Z factor (47), which provides a measure of the reliability of hit selection based on the dynamic range and intrinsic variability of the assay.

**Compounds for HTS.** In addition to a screening assay and instrumentation, drug discovery requires test compounds that can be random (“empirical” discovery), computationally biased to interact with a particular site on the target (“rationally
directed” discovery), or structurally similar to existing compounds with known activity against a target (“lead-based” discovery). The compounds are generally arrayed in sets, referred to as “libraries.” Collections of diverse small molecules (300–600 daltons) are most often used for primary compound screening, in part because they are readily available and amenable to synthetic chemistry to generate combinatorial libraries and engineered structural analogs. Greater than 90% of approved drugs have a molecular size in the range of 200–800 daltons. There are currently >8,000,000 nonproprietary small molecules (ChemNavigator database) available from commercial sources for purchase individually, as user-specified collections, or as preplated sets selected for primary screening applications. Most commercially available compounds have been collected from organic chemistry laboratories worldwide and are generally nonchiral and easily synthesized. Several commercial sources (e.g., ChemBridge, ChemDiv, MayBridge) have assembled collections of diverse prescreened compounds having favorable druglike properties and low cellular toxicity. Typical costs for the purchase of compound collections are US$1.00–2.00 per 0.1–0.5 mg of compound. Compounds are generally delivered as dry powders or as 10 mM stock solutions in dimethyl sulfoxide (DMSO) in 96-well plates or in individual vials in multiwell plate format. Compounds are generally 80 to ≥90% pure and are provided with analytical data such as 1H NMR and mass spectrometry. However, the accuracy, purity, and stability of purchased compounds cannot be assumed, requiring analysis and/or re-synthesis of compounds of potential interest.

A variety of computational algorithms have been developed to predict drug properties from compound structure, such as water solubility, ADME (absorption, distribution, metabolism, elimination), and blood-brain barrier penetration (22, 26). In addition, algorithms have been written to evaluate the diversity of compound collections (14, 41), which is particularly important to the success of a drug discovery program. Although most drug discovery programs utilize collections of synthetic small molecules, there is increasing availability of collections of natural compounds, such as crude organic extracts of plant homogenates and individual compounds purified from extracts (10). Natural compounds can add unique diversity to a compound collection; however, the structural complexity of natural compounds presents a significant challenge in follow-up chemistry for compound optimization.

Generally >50,000 diverse compounds are evaluated in a primary screen to give an acceptable chance of identifying useful active compounds, or hits. Some workers recommend initial screening of no less than 100,000–200,000 compounds and even >1,000,000 compounds. Compounds are most often screened individually (at 5–20 μM final concentration) but are sometimes screened in small groups of 2–10 compounds per assay or larger groups of 100 or more compounds per assay. Assaying individual compounds is more time-consuming and costly than assaying the same compounds in groups but permits testing of higher concentrations and immediate identification of active compounds without having to access and analyze individual compounds from groups.

Our laboratory has collected ~220,000 small molecules, mostly from commercial sources. Stock solutions of original compounds (10 mM in DMSO) are stored at −20°C in bar-coded 96-well plates. Also prepared and stored are mixtures of four (random) different compounds per well (2.5 mM per compound) and several plates of diluted individual compounds (1 mM in DMSO). To minimize time and cost, we usually screen mixtures of four compounds per assay. After confirmation of the activity of hits and completion of initial dose-response studies using the compound mixture, individual compounds are analyzed. The low probability that an individual compound is active or toxic minimizes concerns about false positives/negatives because of toxicity of a single compound or synergy among compounds in a mixture. Various recommendations have been made concerning compound storage conditions to maintain activity, ranging from room temperature storage to −80°C storage with no more than a few freeze-thaw cycles. The general paradigm is to store large amounts of material without solvent, “master plates” at high concentration in DMSO with minimal exposure to freeze-thaw cycles and humidity, and “daughter plates” at working concentrations that are discarded annually. Despite a large body of work on the problem, there are no general conditions that guarantee sample integrity because of the variability of individual compounds.

**Evaluation of hits: selection of leads.** Although compound screening can be challenging and expensive, the primary screen is generally completed in one week to one month, depending on the number of compounds to be tested and the complexity of the screen. The secondary evaluation of hits from a primary screen is a more time-consuming process, the goal of which is to select lead compound(s) for further development. Initial evaluation of hits is designed to identify false positives. Retesting of hits with the original HTS assay is done to confirm activity, followed by target-specific evaluation. For example, in a screen involving target-transfected cells, lack of activity is confirmed in parental/null cells. More definitive and time-consuming “secondary screening” assays are performed to confirm target specificity. For example, electrophysiological analysis of a target ion channel can be done after hits are identified from a primary screening involving ion-sensitive fluorescent dyes or radioisotopes. The structures of confirmed hits are examined to search for common motifs and for initial evaluation of potential medicinal value, which often includes computational “in silico” modeling and determination of physicochemical properties (e.g., solubility, pKa). Prioritization of confirmed hits is done by evaluation of compound potency (concentration-activity measurements) and chemical structure. Prioritization of hits in academic drug discovery varies significantly from industry. Companies may focus on compounds of classes in which they have significant intellectual property holdings rather than compounds with large bodies of competing prior art. In contrast, academic investigators have the freedom to explore chemical space in a more unrestricted manner, which can yield valuable reagents for further study and/or potential partnering opportunities with pharmaceutical companies. Finally, there may be target-specific selectivity issues that require early evaluation. For example, an inhibitor of the CFTR chloride channel may modulate the activities of homologous ATP-binding cassette (ABC) proteins or other chloride channels.

Additional early evaluation of hits involves the examination of the spectrum of activity of sets of structurally related compounds. These studies will define the structure-activity relationship (SAR) for the hit (40). These studies can be executed by purchase of similar existing compounds (SAR by
catalog) and/or by the synthesis of small, carefully designed sets. Hits will generally fall into one of three classes: those with a “flat SAR,” where all compounds in the class have similar activity; “singletons,” where only the original hit has activity; and those with an “evolvable SAR,” where analogs exhibit a broad range of activity that correlates with structural features. Hits with a flat SAR and singletons are often abandoned at this stage, whereas those with evolvable SARs are advanced to the early lead category. Hits that chemically modify a target or act irreversibly are also generally abandoned.

The selection of lead compounds for optimization and more extensive preclinical evaluation requires many additional analyses of confirmed hits, with the timing and depth of the analyses dependent on target biology, assay availability, and other scientific and practical considerations. Initial evaluation of compound toxicity is generally done in cell culture models by using cell viability and proliferation assays and in small rodents by serial evaluation of animal growth and appearance, food/water intake and urine/stool output, and blood chemistries and hematologies. Small animal pharmacology includes analysis of pharmacokinetics (PK) and ADME properties (24). Initial PK is generally done in rats, where blood is sampled serially after intravenous bolus infusion, extracted via liquid-liquid or solid-phase methods, and then analyzed by liquid chromatography/mass spectrometry (LC/MS) or equivalent technologies (32). PK data (blood/serum concentration vs. time) are analyzed to determine distribution and elimination times as well as AUC (area under curve), a measure of total compound exposure. Compound disposition and metabolism in organs and bodily fluids is determined by LC/MS analysis of organ homogenates and fluids such as bile (45). If radiolabeled compound is available, organ distribution can be determined efficiently by autoradiography of whole animal slices or detection of radioactivity in organ homogenates (35). Compound elimination is determined by analysis of urine/stool samples collected by using metabolic cages. The goal of animal pharmacology is to account quantitatively for the distribution, elimination, and processing of administered compound. Issues related to compound administration such as oral bioavailability and absorption mechanisms can also be considered at this stage in drug development, as can analysis of compound specificity (e.g., receptor/ion channel screens), possible drug-drug interactions (e.g., P-450 enzyme screens), mutagenic potential (e.g., Ames test), and toxicity with long-term administration.

Efficacy in animal models and pharmacodynamics (PD) are very important in lead selection. Active compounds in vitro may be inactive in vivo for many reasons, such as inadequacy of an in vitro screen to recapitulate in vivo target biology, rapid in vivo metabolism or elimination, or poor penetration into target tissues or cellular sites within a tissue. Evaluation of efficacy in animal models is of course target specific. For example, serum cholesterol concentration might be a good endpoint assay for an inhibitor of ileal bile acid transport, as might electrocardiography for inhibitors of cardiac ion channels. Activators of ΔF508-CFTR could be evaluated electrophysiologically in a cystic fibrosis mouse model over the short term by measurement of transepithelial potential differences or over the longer term by mouse survival and prevention of gastrointestinal pathology. PD is the kinetics of a biological response after compound administration (as opposed to compound concentration in PK). Because of tissue differences in compound access and metabolism, the magnitude and kinetics of a PD response are assay dependent. Furthermore, there may be significant differences in PK vs. PD because of the slow/delayed kinetics of a biological response, the presence of active metabolites, slow/irreversible compound dissociation from the target, or complex in vivo processing such as enterohepatic recirculation. It should be recognized that imperfect specificity, pharmacology, and efficacy profiles can be tolerated for potential lead compounds because substantial improvements are possible during lead optimization. However, some types of failures, particularly poor permeability for a drug intended for oral administration, hepatotoxicity, or induction of markers of long QT syndrome, are correlated with lead failure and thus reduce enthusiasm for follow-up work on a potential lead compound.

Lead optimization by synthetic/medicinal chemistry. The goal of lead optimization is to generate structural analogs of lead compounds that are optimized in their potency, specificity, and pharmacology/ADMET (ADME + toxicity) properties for entry into clinical trials. Lead optimization generally involves iterative rounds of synthetic organic chemistry and compound evaluation that can take from months to years and involve the efforts of a team of synthetic organic chemists. The starting point for lead optimization is the collection and analysis of SAR data. As mentioned above, initial SAR data can be generated efficiently by analysis of existing structural analogs obtained from commercial sources. The first synthetic efforts will generally focus on systematic exploration of tolerated functional groups and steric character in all areas of the candidate molecule. Later rounds will build the initial analysis and are often targeted at solving particular problems. One important goal of compound optimization is to improve potency, since high potency minimizes dose and hence improves specificity and reduces toxicity. Potencies (expressed as IC₅₀) in the range of 100 nM or better are desired, though much lower potency can be tolerated if specificity is high, toxicity is low, and the mode of administration can accommodate the required dose.

Several approaches are available to maximize the utility of SAR information for directed acquisition and synthesis of structural analogs to improve compound potency. Advice from experienced medicinal chemists is of considerable practical value to identify undesirable structural features that may result in failure because of toxicity, poor biological stability, immunogenicity, or mutagenic potential (42). Consideration of empirical information about drug successes and failures can be quite helpful in directing synthesis efforts. In terms of utilizing SAR data to accelerate compound optimization, visual inspection of SAR data reveals many important structural features associated with activity. Three major computational methods have been used in lead optimization: rational structural design, pharmacophore analysis, and quantitative structure-activity relationship (QSAR) analysis. The three may be used individually or in combination. Rational design methods generally use a high-resolution structure of the target to direct the synthesis of new analogs. The process often involves generation of a very large in silico library of potential derivatives and use of computational docking methods to select derivatives that may interact with the target on the basis of shape complementarities and charge placement. However, while intellectually appeal-
ing, there have been few examples of success when this strategy is used by itself. Pharmacophore methods involve definition of the minimal unit that leads to activity (usually a combination of hydrogen bond donor/acceptors, hydrophobic groups, and other functional groups) in a three-dimensional space (2, 15, 23, 33). The consensus pharmacophore is then used to examine the allowed placement of groups in a set of candidate compounds. Pharmacophore analysis can be carried out without structural information and is most useful in identifying new compounds with a desired activity based on a three-dimensional similarity to early leads. As an example, screening of a combinatorial collection of flavones yielded a class of 7,8-benzoavones that were substantially more potent CFTR activators than existing flavones (13). Analysis of SAR data from a targeted set of synthesized benzoflavones yielded the pharmacophore model shown in Fig. 3A (36), where key structural/polarity features of the pharmacophore are indicated. Interestingly, the structures of many of the novel CFTR activators identified by HTS (28) fit well in the flavone-based pharmacophore model, suggesting a common binding site. The final approach is to establish QSAR models that relate calculated physicochemical properties of molecules, rather than strictly structural characteristics, to activity (1, 34). This type of modeling is particularly important in directing modifications to PK and PD parameters. QSAR modeling requires a set of structurally related compounds with a wide range of activities, ideally 1,000-fold variation in activity, which is often a difficult requirement to meet.

Alternative strategies have been developed to predict the activity of structural analogs by utilizing a data set containing inactive analogs and active analogs having a limited range of potencies. As an example of a Bayesian statistical approach to predict compound activity, our laboratory recently identified small molecule correctors of defective ΔF508-CFTR chloride channel gating from a primary screen of 100,000 diverse compounds (46). Compound optimization was focused on substituted tetrahydrobenzothiophenes because of their good medicinal properties and evolvable SAR with potencies of <1 μM for many hits identified in the primary screen. As a first step in compound optimization, 3,025 commercially available tetrahydrobenzothiophenes were assayed, yielding 40 active compounds. Seven compound parameters were compared, including molecular weight, surface area, polar surface area, number of hydrogen bond donors, number of hydrogen bond acceptors, AlogP (related to octanol:water partitioning), and a structural/connectivity index. Figure 3B summarizes the physical properties of active tetrahydrobenzothiophenes satisfied by >70% of active compounds. The number of hydrogen bond donors and acceptors was low (≤3 each), and the overall polar surface (72 ≤ Å² ≤ 98) and AlogP (2.3–3.6) fell within a narrow range. After appropriate statistical normalizations, the seven computed parameters for each compound were incorporated into a single dimension, or “model score.” A Bayesian learning approach utilizing randomly partitioned subsets of the data was applied to distinguish active vs. inactive compounds and was cross-validated by using data subsets that were not included in the model (46). Figure 3C shows that active vs. inactive compounds were well distinguished on the basis of the single model score. Furthermore, a “common structural features” analysis of active compound indicated important structural features (Fig. 3D), including 1) the presence of a 4,5-fused tetrahydrobenzothiophene with the fused ring being a 6- or 7-member aliphatic ring, 2) the presence of an unsubstituted carboxamide at the 3-position, and 3) a high population of aromatic amides at the 2-position. The computational assessment of the likelihood that a new analog will be active can substantially accelerate compound development, for example, in the “virtual screening” of large commercial compound collections for active analogs before selection of compounds for purchase.

All of the described computational methods can be implemented in an academic environment by using commercially available software.
available programs from a variety of vendors. In general, none of the methods require extensive code generation or high levels of computational expertise, and most can be run on standard UNIX or PC machines.

**EXAMPLE OF ACADEMIC DRUG DISCOVERY:**

**CFTR MODULATORS**

CFTR is a well-validated target for drug discovery. Activators of mutant CFTRs causing cystic fibrosis have potential applications in cystic fibrosis therapy, and inhibitors of wild-type CFTR are predicted to be effective in the therapy of secretory diarrheas and in the creation of large animal models of cystic fibrosis (39). Because the principal function of CFTR is plasma membrane cAMP-stimulated halide transport, we focused on the development of cell-based assays of halide transport suitable for HTS. It was reasoned that direct measurement of cytoplasmic halide (chloride or iodide) concentration would provide a more direct readout of CFTR function than surrogate readouts such as membrane potential or pH changes, which could produce many false negatives and positives in compound screening. Fluorescent indicators of cytoplasmic halide concentration would provide a more direct readout of CFTR function than surrogate readouts such as membrane potential or pH changes, which could produce many false negatives and positives in compound screening. Fluorescent indicators of cytoplasmic halide (chloride or iodide) concentration would provide a more direct readout of CFTR function than surrogate readouts such as membrane potential or pH changes, which could produce many false negatives and positives in compound screening.

We reasoned that an expressed fluorescent protein would be superior to chemical fluorophores in screening applications because loading/washing steps would not be required and dye leakage would not occur. Green fluorescent protein-based halide sensors were developed for this application, the first being the yellow fluorescent protein (YFP) mutant YFP-H148Q (17). Fluorescence titrations of purified recombinant YFP-H148Q indicated a pKa of ~7 in the absence of chloride that increased to ~8 at 150 mM chloride (Fig. 4A). At pH 7.5, YFP-H148Q fluorescence decreased with increasing chloride and iodide concentration with 50% quenching at ~100 mM chloride and 21 mM iodide. A static quenching mechanism predicting chloride binding to YFP-H148Q was established by biophysical methods including fluorescence lifetime and stopped-flow kinetic analysis. A detailed kinetics model incorporating these quantitative results indicated four equilibria involving YFP-H148Q protonation and chloride binding (Fig. 4B). Subsequent crystallographic studies of YFP-H148Q in the presence of iodide confirmed a discrete binding site for halides (44). YFP-H148Q was also expressed in tissue culture cell lines to establish its utility in cell-based assays of chloride concentration (17). YFP-H148Q fluorescence was observed throughout the cell cytoplasm and nucleus. Calibration experiments in which ionophore-treated cell cultures were used indicated that the pH and chloride (Fig. 4C) sensitivities of YFP-H148Q were similar in cells and aqueous solutions. The utility of YFP-H148Q to follow CFTR-mediated halide fluxes was established on the basis of the ability of CFTR to conduct chloride and iodide. Large changes in fluorescence intensity of ~50% were observed in chloride/iodide exchange protocols (Fig. 4D). In follow-up studies, substantial improvements in YFP halide sensitivity and brightness were made by screening YFP mutational libraries (11). Screening of ~1,000 YFP mutants yielded YFPs with very different chloride and iodide affinities compared with YFP-H148Q. Mutants V150T, I152L/Y, V163T/L, V163A/F165Y, and V163T/F165Y had Kd values for iodide of ~15 mM in the initial screen. The I152L mutant has been particularly useful in screening applications because of its strong cellular expression, bright fluorescence, and very different dissociation constants for chloride vs. iodide at cytoplasmic pH of 85 and 2 mM, respectively, permitting the use of a chloride/iodide exchange protocol.

Assay development also required selection of cell lines for expression of halide-sensing YFPs together with wild-type or mutant CFTRs (12). The requirements of a cell line for CFTR screening applications include rapid growth utilizing relatively inexpensive media, good adherence to plastic multiwell plates.
during solution washing/exchange, low CFTR-independent chloride permeability, and bright and stable YFP/CFTR expression. In addition, an epithelial cell type is desirable to recapitulate the normal CFTR expression pattern and because epithelial cells forming electrically tight monolayers permit efficient secondary analysis of hits by short-circuit current analysis. Fisher rat thyroid (FRT) cells were chosen after multiple cell lines were screened for these characteristics. Clones of brightly fluorescent, doubly transfected FRT cells were selected and expanded for large-scale screening.

Figure 5A shows the strategy used for identification of CFTR inhibitors. FRT cells coexpressing human wild-type CFTR and the YFP-based halide sensor were stimulated by a CFTR-activating cocktail and then subjected to an iodide gradient (27). The rationale for using multiple CFTR activators was the prediction that compounds with inhibitory activity would likely act directly at the CFTR anion pore or another key site, rather than indirectly at upstream or downstream sites in the CFTR activation/deactivation pathway. Iodide addition produced a prompt decrease in cell fluorescence after CFTR activation (Fig. 5B). Inhibitors (“active compounds”) were identified from a reduction in the (negative) fluorescence slope. The best inhibitor identified by screening and follow-up optimization was the 2-thioxo-4-thiazolidinone compound CFTRinh-172 (Fig. 5C, top). CFTRinh-172 inhibited CFTR function at submicromolar concentrations (Fig. 5C, middle and bottom), ~500 times more potent than the previous best inhibitor, glibenclamide, studied under similar conditions. Electrophysiological analysis indicated voltage-independent inhibition of CFTR chloride conductance by CFTRinh-172 with prolonged mean channel closed time and without altered unitary conductance. CFTRinh-172 did not affect the activities of several other chloride channels (calcium- and volume-activated) or ABC transporters (multidrug resistance protein-1, sulfonylurea receptor). Rodent pharmacology studies indicated low toxicity, a large volume of distribution with slow elimination by renal glomerular filtration, small metabolism, and enterohepatic recirculation with accumulation in bile and intestine.

The anti-diarrheal efficacy of the thiazolidinone CFTRinh-172 was tested in a mouse closed ileal loop model (37). Injection of cholera toxin into ileal loops produced measurable fluid secretion over 6 h after a slow onset. A single intraperitoneal injection of CFTRinh-172 reduced fluid accumulation by ~90%, with 50% inhibition at ~5 μg of CFTRinh-172 and a duration of action of 6–10 h. Orally administered CFTRinh-172 was effective in blocking intestinal fluid secretion after oral cholera toxin in an open-loop model. CFTRinh-172 also inhibited fluid secretion induced by Escherichia coli STa toxin, as well as cAMP- and cGMP-stimulated chloride currents in human intestine. The anti-diarrheal efficacy and favorable pharmacology of CFTRinh-172 in these initial studies suggests the potential utility of thiazolidinones in reducing intestinal fluid secretion in cholera and Traveler’s diarrhea.

Similar screening strategies were used to identify activators of wild-type CFTR as well as “potentiators” (correctors of defective gating) and “correctors” (correctors of defective cellular processing) of ΔF508-CFTR. In an initial project to discover new classes of CFTR activators with improved potency and selectivity over existing compounds such as flavones, a small molecule collection was screened with the use of the CFTR/YFP-expressing cells described above. Primary screening consisted of short-term stimulation with 10 μM test compound and 0.5 μM forskolin followed by iodide challenge. The screen yielded 57 strong activators (greater activity than reference compound apigenin at 50 μM), most of which were unrelated in chemical structure to known CFTR activators. Secondary analysis revealed three compounds with submicro-
molar potency that activated CFTR indirectly by increasing cellular cAMP and fourteen compounds that activated CFTR without cAMP elevation or phosphatase inhibition, suggesting direct CFTR interaction.

Potentiators of ΔF508-CFTR gating were identified by screening FRT cells that stably expressed ΔF508-CFTR and the halide-sensitive indicator YFP-H148Q/I152L (46). A cell clone was selected that showed consistent expression of ΔF508-CFTR protein at the plasma membrane when incubated at 27°C for 24 h, because ΔF508-CFTR is a “temperature-sensitive mutant” in which growth at low temperature permits plasma membrane targeting (5). One hundred thousand small molecules were screened for activation of halide transport in cells incubated at 27°C for 24 h to permit ΔF508-CFTR plasma membrane targeting, followed by 15-min incubation with forskolin and test compounds. The screen and subsequent secondary analysis yielded six novel chemical classes of ΔF508-CFTR potentiators, with the most potent belonging to the tetrahydrobenzothiophene class, as discussed above (see Fig. 3D). To screen for correctors of defective ΔF508-CFTR cellular processing, the ΔF508-CFTR/YFP-H148Q/I152L-expressing FRT cells were incubated with test compounds for 18 h at 37°C and then assayed for iodide efflux in the presence of a strong ΔF508-CFTR potentiator and cAMP agonist (43). Initial screening and follow-up optimization yielded several classes of compounds that at micromolar concentrations restored halide conductance to cells with greater efficacy than growth at low temperature. The challenge will be to establish the mechanisms of ΔF508-CFTR rescue and to select compounds with good specificity and efficacy in vivo for further development. ΔF508-CFTR potentiators and correctors may be of potential utility for therapy of cystic fibrosis caused by the ΔF508 mutation.

PERSPECTIVE

Despite challenges, drug discovery programs can be established and maintained in an academic setting. Academic drug discovery programs can generate unique reagents for hypothesis-driven research and drug candidates for targets of limited commercial interest. There is increasing recognition by the National Institutes of Health and other funding agencies of the importance of drug discovery as a key component of translational research and of the unique contributions that can and should be made by academic investigators.

ACKNOWLEDGMENTS

We thank Drs. Devin Binder, R. Kip Guy, David MacDonald, and Frank Szoka for critical reading of this manuscript and many helpful suggestions.

GRANTS

This work was supported by Drug Discovery and Research Development Program Grants from the Cystic Fibrosis Foundation and by National Institutes of Health Grants HL-73856, HL-59198, EB-00415, EY-13574, and DK-35124.

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