Application of High-Throughput, Molecular-Targeted Screening to Anticancer Drug Discovery

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Abstract: Increasing insight into the genetics and molecular biology of cancer has resulted in the identification of an increasing number of potential molecular targets for anti-cancer drug discovery and development. These targets can be approached through exploitation of emerging structural biology, "rational" drug design, screening of chemical libraries, or a combination of these methods. In this article we discuss the application of high-throughput screening to anti-cancer drug discovery, with special reference to approaches used at the U.S. National Cancer Institute.

INTRODUCTION

This article provides an overview of the application of high-throughput, molecular targeted drug screening to anticancer drug discovery. The review provides a brief historical perspective on anticancer drug discovery and describes recent developments which have enabled a fundamental change, molecular targeting, in cancer treatment. References to recent reviews and selected new technologies are included in the text. For recent general reviews of high-throughput screening as practiced in the pharmaceutical industry, the reader is referred to Cox *et al.* [1] and Landro *et al.* [2]. Illustrations of the concepts and application of molecular targeted screening to anticancer drug discovery are provided from current efforts within the National Cancer Institute's Developmental Therapeutics Program.

HISTORICAL PERSPECTIVE ON ANTICANCER DRUG DISCOVERY

One of the first large-scale, systematic approaches to anticancer drug screening was organized in the United States at the National Cancer Institute (NCI) in the mid-1950's [3]. This Congressionally mandated effort responded to the growing public awareness of therapeutic potential in the new field of cancer chemotherapy and the knowledge that the pharmaceutical industry was not yet investing research resources in this area. Early models for screening were based on transplantable tumor models in rodents and came to feature drug sensitive mouse leukemias as primary screens [4]. As the pharmaceutical industry joined the anticancer drug discovery effort, companies tended to adopt similar screening and detailed drug evaluation methods. These were described in detail by NCI investigators to facilitate this process [5]. While these early discovery efforts, based on antitumor activity in living animal hosts, yielded success in treatment of several diseases, most notably childhood leukemia, the limited successes in treatment of the common adult solid tumors led to investigation of alternative drug discovery models including *in vitro* screens using human tumor [6,7] or cell line models [8,9]. The NCI 60 tumor cell line screen, initially implemented as an empirical model designed to select compounds based on exploitable features of particular solid tumor types, proved to be a powerful tool for obtaining insight into mechanisms of growth inhibition and cell kill [10-12]. Integration of information regarding molecular phenotypes of the screening panel cell lines, including patterns of gene expression derived from gene arrays [13,14] has provided insight into potential molecular mechanisms which could not be recognized previously. For a more extensive discussion of the origins of cancer chemotherapy and the involvement of the NCI, see Shoemaker and Sausville [15].

ADVENT OF MOLECULAR MEDICINE

The increasing understanding of cell surface receptors and other molecular targets for non-cancer indications served as the basis of pharmaceutical industry efforts towards molecular targeted drug screening. Success in discovery of

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agents active in cardiovascular, anti-inflammatory, and anti-infective areas [16] with well-characterized molecular targets has fueled technological developments enabling new approaches to drug discovery. Technology for molecular modeling and drug design, coupled with increased availability of structural information regarding molecular targets and the completion of the human genome project [17,18] have created major new opportunities for drug discovery in all areas of human disease. Target-based screening in the pharmaceutical industry resulted in the discovery of Gleevec, a kinase inhibitor with unique therapeutic activity in chronic myelogenous leukemia [19,20] recently approved by the U.S. Food and Drug Administration. This, and additional examples of targeted anti-cancer drug development by the pharmaceutical industry are discussed in additional detail later in this article.

TECHNOLOGY ENABLING HIGH-THROUGHPUT SCREENING

The fundamental requirements for high-throughput screening are the availability of chemical libraries for testing and the capacity to test them in a rapid fashion.

Pharmaceutical companies have developed large collections of chemical compounds. Some of these collections, developed over many years, and in some cases recently expanded through technology for combinatorial chemistry and parallel synthesis, are of very large size (many hundreds of thousands of samples). Synthesis of many analogs during lead optimization of successful products, in some instances, has been thought to bias the range of chemical diversity in these corporate collections. Computational tools offer a variety of means for assessing diversity in chemical libraries. A considerable number of such methods currently exist for electronic coding of molecular structures. A comprehensive listing of most of these chemical descriptors can be found in the *Handbook of Molecular Descriptors*, edited by R. Todeschini and V. Consonni [21]. Numerous early efforts were made to use selected molecular descriptors to develop diverse as well as focused libraries of compounds [22-28]. The primary aim of these efforts was to define libraries that could be optimized for testing against a specific molecular target and by doing so improve screening efficiency [2,21]. For additional information on this topic see [29-31]. While these electronic descriptors are a convenient way of defining chemical structures, the selection of which method to use, and the underlying features coded in different molecular descriptors will critically determine the composition of a focused library [32]. An additional component of these analyses involves the statistical methods used to define structural classes. A large number of approaches are currently available that involve supervised and unsupervised clustering methods [33-37]. While each of these methods has a sound foundation in mathematical statistics, their applications to large compound libraries remains a difficult task [21].

Through four decades of collection, of largely donated materials, the NCI has amassed samples of more than 500,000 synthetic compounds. The range of chemical diversity in this library has recently been assessed and compared to other available libraries [32]. In this analysis, the NCI library was found to contain a large number of compounds (ca. 200,000) not represented in other libraries, including the Available Chemical Directory. Software tools have been applied to the NCI library for a variety of purposes including generating a subset of compounds intended to maximally represent the three-dimensional chemical diversity in the whole library. This set of approximately 2,000 compounds, the NCI "Diversity Set" is available to the research community as described on the DTP website (http://dtp.nci.nih.gov) and can be quite useful for HTS assay development and initial characterization. In certain instances, this small library has yielded potentially significant drug leads in HTS. Stephen, *et al.* [38] have identified potent HIV-1 nucleocapsid binding compounds which have been shown additionally to have anti-HIV activity in a cell-based assay. Lazo, *et al.* [39] have recently identified novel inhibitors of protein phosphatase Cdc 25. An additional compound set, the "Training Set", has been created which provides representatives of the major mechanistic classes of standard anticancer drugs, as well as a variety of compounds known as specific inhibitors of enzymes or signaling pathways. As described subsequently, this small collection of approximately 250 samples can be useful for initial characterization of performance of new HTS assays prior to scale-up to higher throughput screening.

Approximately 140,000 compounds available in sufficient quantity and free of confidentiality constraints have been prepared in 96-well master plates. An analysis of the chemical structural features of this collection has been completed. Initially a variety of electronic methods were used to describe the chemical features of these compounds [21]. A comprehensive analysis, using different chemical descriptors, found that amongst five different classes of descriptors the Daylight Chemical form (Daylight Chemical Systems) proved superior (manuscript in preparation). In this determination a heuristic criterion was adopted that measured the success of using different chemical descriptors to correctly classify compounds into previously determined classes based on their putative biological effect [40].

Based on the Daylight Chemical Descriptors a self-organizing map (SOM) was completed for the plated library. Cluster analyses of large numbers of compounds (N=140,000) based on a relatively large number of chemical descriptors (M=2048) represents a considerable challenge for most clustering methods. As an example, the SOM map shown here required nearly 6 CPU days to complete on a modern high-speed computer. As illustrated in Figure **1**, the results of this analysis find that the chemical space can be determined by a 63X41 SOM map to comprise 2583 cluster classes. Thus, an initial data reduction is achieved that maps 140,000 compounds into an approximately fifty-fold smaller space. Each of the clusters is comprised of structurally similar compounds with Tanimoto coefficients greater than 0.7. This widely used similarity measure assigns a value from 0 to 1.0 (where 1.0 represents identity) based on the extent of commonality of structural descriptors [41,42]. The gray color scale on this map is a measure of the distance between map clusters. The darkest regions represent areas where sets of chemically similar compounds appear, while the lighter regions identify chemically distinct subsets.

Thus, the SOM analysis provides an opportunity to easily identify focused (i.e. similar) as well as de-focused (i.e. dissimilar) subsets of compounds.

As an example of how the SOM map partitions chemical space, the locations of clusters containing at least one member of the NCI's Diversity Set are shown as yellow open hexagons in Figure **1**. It should be noted that the compounds included in the Diversity Set were selected by Dr. Daniel Zaharevitz of the NCI Developmental Therapeutics Program using a Chem-X program with the goal of generating a set of compounds diverse in terms of three-dimensional pharmacophores (http://dtp.nci.nih.gov/branches/dscb/diversity_explanation.html). In the context of the SOM analysis of the plated library of compounds, the Diversity Set spans the entire map. Also evident from this analysis is the observation that, while the Diversity Set appears to be truly diverse, in terms of extent of map coverage, many of the map clusters contain relatively large numbers of compounds from the Diversity Set. As an

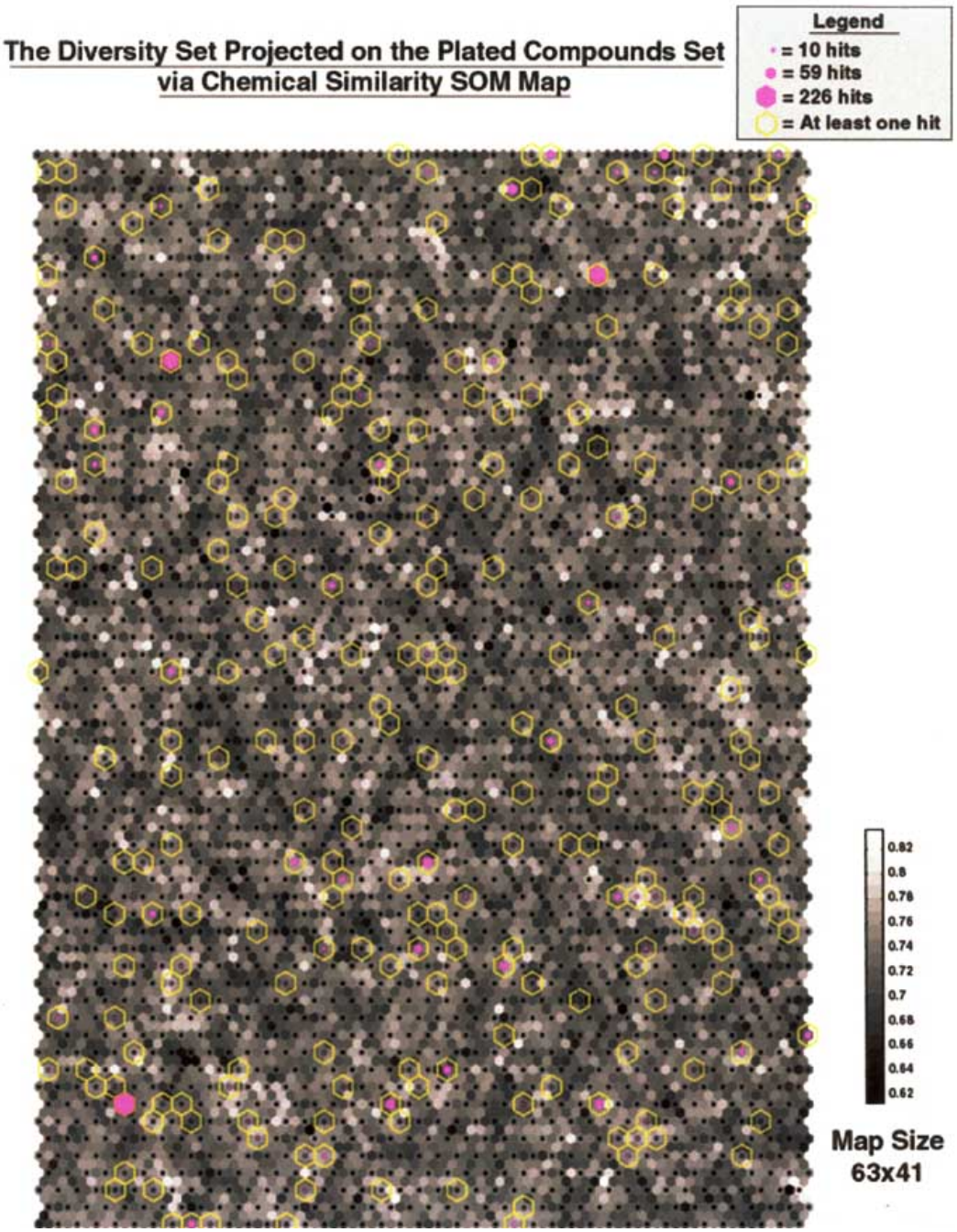

Fig. (1). Results of SOM analysis of 140,000 compounds from the NCI chemical library. Diversity Set members are projected onto the larger library as indicated in yellow open hexagons, for clusters with single compounds or in magenta for clusters with multiple members. The compound densities are indicated on the Figure for these latter clusters.

example, three clusters contain over 200 Diversity Set compounds, thus accounting for nearly forty percent of this library. The magenta hexagons displayed on Figure **1** locate where the larger subsets of compounds from the Diversity Set are located. Evident from this analysis is the result that, while the Diversity Set is distributed across the entire chemical space of the plated library set, over 89.8 percent of the map clusters lack a structurally similar member from the Diversity Set. We are currently engaged in selection of a second generation "Diversity Set" which is optimized for coverage of the chemical space defined in this SOM analysis but which retains a defined level of chemically similar compounds. In practical terms, the presence of some chemically similar compounds adds value to the set for use in initial characterization of new HTS assays. This value lies in the opportunity to identify related compounds as active, thus demonstrating coherence in the screening data.

Natural product extracts provide, perhaps, the greatest possible range of chemical diversity and historically have been a major source of useful drugs. Drug discovery based on such libraries requires a process of bioassay-directed isolation and structure elucidation which can represent a daunting task. "Dereplication", the process of identifying and eliminating known compounds from the discovery process, requires a great deal of skill and utilization of chemical informatics. The development of sensitive LC/MS equipment has greatly facilitated this process by allowing precise molecular weights to be associated with biologically active samples. This, and various spectral data can, in many instances, allow rapid identification of previously known compounds and allow investigators to focus on novel drug leads. Through more than a decade of world-wide, contract-based collections NCI has amassed a library of approximately 70,000 paired aqueous and organic extracts from a wide range of natural sources, including plants, marine organisms, microbial cultures, etc. [43]. This library has been used by both extramural and intramural NCI investigators to isolate many novel molecules with antitumor [44] and anti-HIV activity. A comprehensive listing of compounds isolated during more than a decade of work by intramural NCI investigators can be found on the web at: http://home.ncifcrf.gov/mtddp/catalog.html. A number of independent extramural investigators have reported isolation and characterization of anti-tumor drug leads from this extract library [45-47]. Use of crude natural product extract libraries for HTS presents a variety of challenges. For example, tannins, frequently present in plant extracts can cause cross-linking of proteins in cell-free assays and obscure activity of potentially interesting constituents. Cell-based assays tend to be more tolerant of these effects, but may be associated with characteristic artifacts which must be recognized and resolved [48].

Organization of large chemical libraries into physical formats useful for screening has generally entailed the transfer of small amounts to multi-well plates using automated liquid handling equipment. Similar automation equipment, interfaced to microplate readers and data acquisition systems, provides the basis for operation of high-throughput screening assays. "Homogeneous assays" employing various novel technologies which simplify manipulation of assay plates have been introduced to support very rapid testing of these large chemical libraries [49,50]. Several such assays are described below.

ANTICANCER DRUG TARGET IDENTIFICATION AND VALIDATION

The ultimate validation of a drug target rests on the demonstration of a clinically useful therapeutic effect mediated by drug interaction with the target. Thus, pharmacodynamic evaluation of drug effects has become an integral feature of molecular targeted drug development. Gleevec targets the Bcr-Abl kinase uniquely present in chronic myelogenous leukemia and has been extensively studied from the pharmacodynamic standpoint in preclinical models [51]. This kinase may currently be viewed as representing the best validated molecular target in the new era of cancer drug discovery and development [19,20,52]. Other targets of potential importance for solid tumor therapy have been extensively studied by the pharmaceutical industry. Ras-mediated cell signaling offers multiple potential targeting points [53] and has yielded drug candidates directed toward farnesylation, geranyl-geranylation, and other key pathway points [54-57]. The Ras-directed farneslyation inhibitors are useful examples of how agents directed at one target may actually exert effects on other targets (farnesylated proteins) [58]. Thus, a molecular targeting strategy must consider how to focus on the "molecular essence" of a target function to maximize the likelihood that a drug will not have a diverse range of effects.

Support for *in vivo* antitumor action at the level of the respective molecular targets are continuing to emerge [59]. Iressa is currently under clinical evaluation as an inhibitor of the epidermal growth factor receptor [60]. Emerging evidence for clinical activity, coupled to target modulation, supports the validity of this target. Evidence of clinical activity of prior therapeutic approaches to this target, notably the development of anti-EGF receptor monoclonal antibodies, further support the validity of this target [61-63]. A kinase inhibitor directed towards the vascular endothelial cell growth factor receptor tyrosine kinase has recently been reported as a novel approach to inhibition of tumor angiogenesis [64].

The availability of the human genome, projects such as the Cancer Gene Anatomy Project [65,66], and a growing number of computerized approaches to exploitation of this information provides a very large number of potential targets for drug development. In addition to the kinases discussed above, hundreds of additional kinases are coded in the human genome [67] some of which may function as important regulators of signaling pathways potentially important in cancer.

Preclinical approaches to molecular target validation include creation of dominant negative constructs, gene knockouts, and transgenic mice. Genetic demonstration that loss of function is associated with a reduction in tumor formation or growth rate has been taken as support for the validity of a putative drug target. At the cellular level gene expression can be inhibited by antisense or ribozyme

treatment. Recently technology has been introduced to selectively destroy individual proteins in individual cells by laser ablation [68]. This technology could potentially be applied to probing the function of many proteins. Expression of a potential target gene in a transgenic mouse can provide an *in vivo* model for evaluation of potential therapeutic strategies.

DEVELOPMENT OF HIGH-THROUGHPUT SCREENING ASSAYS

There are many possible approaches to development of high-throughput screening assays. These range from highly innovative, automated technologies involving miniturazization of assays to nanoliter volumes [49,69] and specialized assay endpoints [50] to relatively ordinary technologies and 96-well assay plates. The suitability of a particular technology is highly dependent of the nature of the target addressed by the assay. A generalized flow-chart for assay development is shown in Figure **2**. This chart emphasizes the importance of characterizing and standardizing assay components, and characterizing assay performance, particularly reproducibility. The compound sets described above are readily applied to this process in a hierarchical fashion which establishes a solid foundation for high throughput screening campaigns.

Molecular-targeted HTS assays can be generally divided into cell-free and cell-based assays. As summarized in Table 1, these approaches are associated with various characteristic advantages and disadvantages. Cell-free assays can be very simple, precise, readily automated, compatible with very high-throughput, present molecular targets in the absence of confounding variables and thus yield "hits" which unambiguously affect the molecular target. Functional screens for enzyme inhibitors require specialized buffers and reaction components, such as ATP in kinase assays, which can dramatically affect performance of the screen. Cell-based assays present targets within a relevant cellular context and provide some modeling of transport and serum protein binding phenomena. However, cell-based assays potentially offer multiple points of molecular action and thus yield "hits" which require additional testing to define the nature of their action against the target.

Data processing and quality control are major features of HTS. Several useful references are available on this topic [70,71]. A simple, but very useful statistical test for assay quality, the Z' factor has been described recently [70]. This factor addresses both signal-to-noise and reproducibility issues in a single statistic. We routinely evaluate this statistic during development of HTS assays, to ensure adequate assay performance, and during conduct of screening campaigns, to flag individual assay plates which may lie outside of performance limits.

Table I. Comparative Features of Cell-Free and Cell-Based Molecular-Targeted Screening Models

Cell-Based Assays	Cell-Free Assays
● Present targets in context	● Simple
● Include membrane	● Precise
● Include cellular metabolism	● Amenable to very HTS
● Model serum binding	● "Hits" require study in cell-based assays
● "Hits" require mechanistic characterization	

In the Developmental Therapeutics Program of the NCI we have undertaken development of infrastructure for both cell-free and cell-based HTS assays with the goal of establishing a versatile core screening operation capable of pursuing multiple concurrent HTS campaigns addressing a variety of molecular targets identified and validated primarily by investigators in the extramural cancer research community. A peer-review system for extramural access to this and other DTP drug discovery resources has recently been established and is described at: http://dtp.nci.nih.gov.

The general approach to development of HTS that we have adopted is to utilize "off-the-shelf" liquid handling machines and plate reading instruments. As delivered from suppliers, this equipment is capable of accurately pipetting volumes as low as 2 microliters into commercially available microculture plates at densities of up to 1536 wells per plate. We have elected to utilize this equipment in a semi-automated modular workstation approach as opposed to a fully automated robotic laboratory to maximize flexibility in changing technologies and screening targets. This semi-automated approach, in which technicians manipulate stacks of bar-coded assay plates from one workstation to another, is

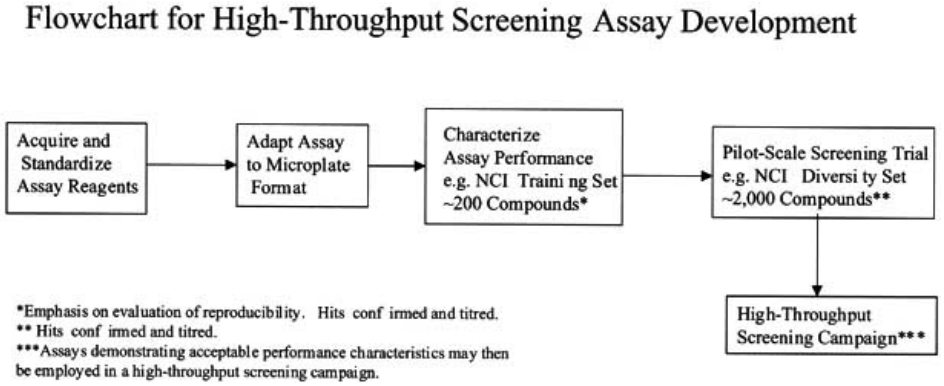

Fig. (2). Generalized flow-chart for high-throughput drug screening assay development.

easily capable of meeting or exceeding our minimum HTS throughput of 10,000 samples per month. Indeed, for certain assays, as described below, a throughput five times higher can be readily achieved.

Cell-Free HTS Assays

Among the simplest cell-free assays are those that directly assess interaction of proteins with peptides or nucleic acids. These can be conducted in simple aqueous buffers either as ELISA-style assays with target protein immobilized on assay plate surfaces or in solution, using fluorescence polarization (FP) or other detection technology. As an example of the former approach, we have conducted HTS campaigns for inhibition of binding of the HIV-1 nucleocapsid protein/nucleic acid binding [72]. Screening of ca. 18,000 samples has yielded a number of confirmed active inhibitors, including one notable series of molecules with low-nanomolar binding affinity for the nucleocapsid protein and clear anti-HIV activity in cell-based assays [38].

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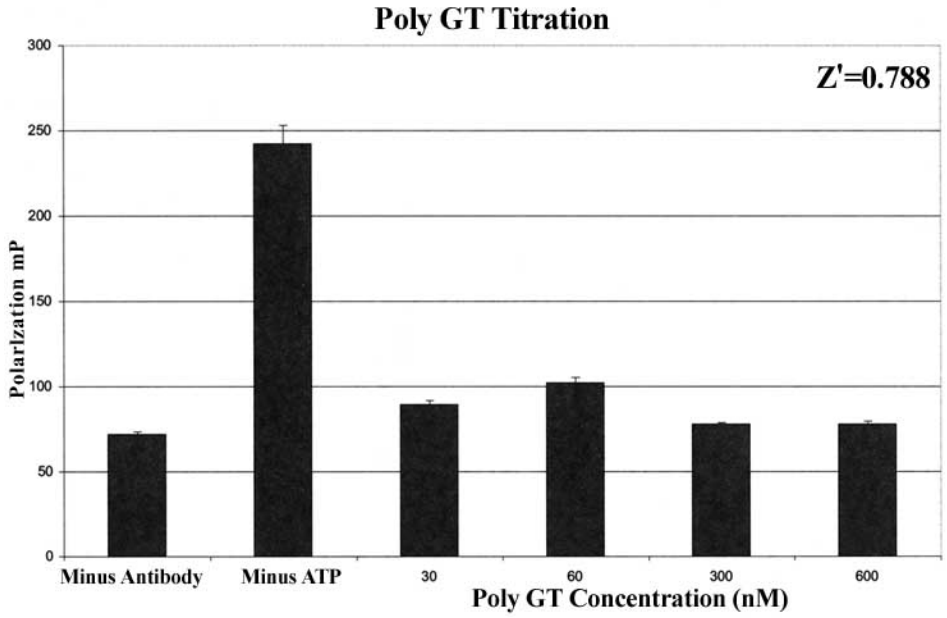

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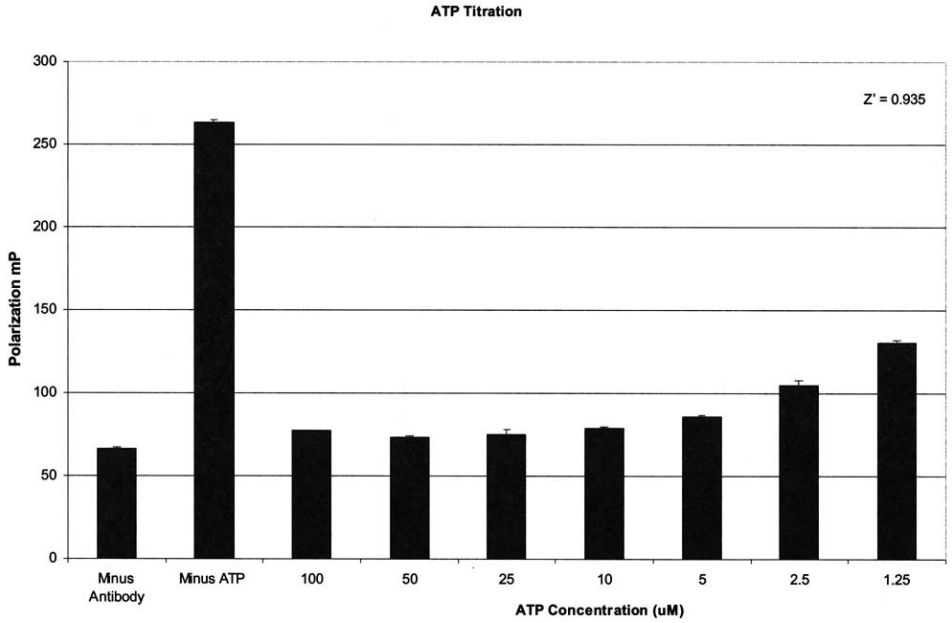

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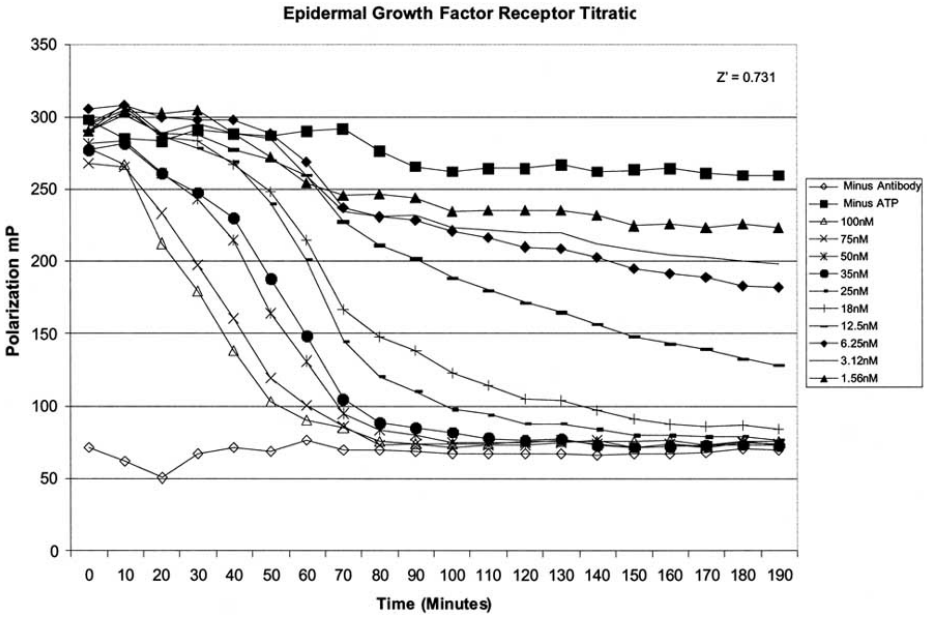

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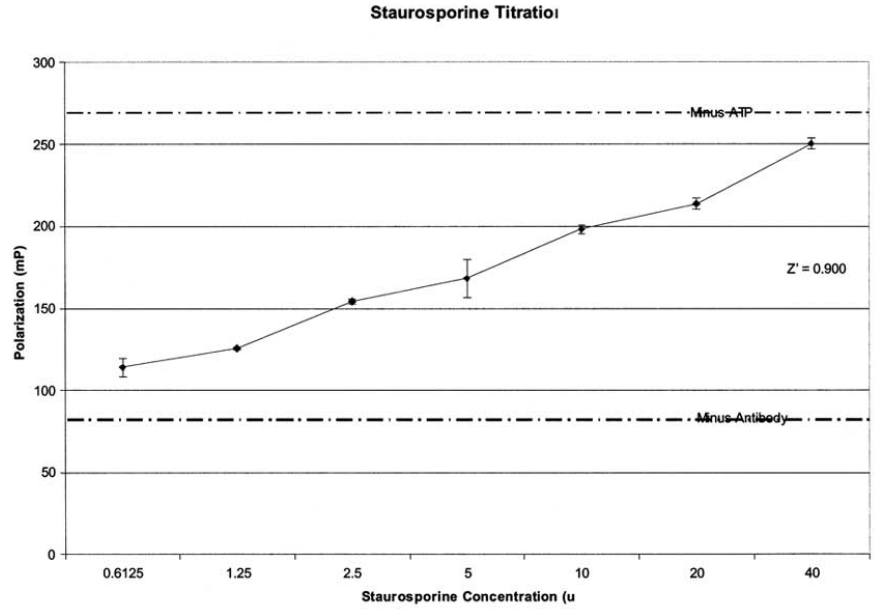

Fig. (3). Optimization of major parameters for an EGF-receptor tyrosine kinase assay using fluorescence polarization technology. Figure **3A**: Titration of Poly GT. Figure **3B**: Titration of Poly ATP. Figure **3C**: Titration of epidermal growth factor receptor tyrosine kinase. Kinetic readings were taken at 10 minute intervals for 180 minutes. Figure **3D**: Titration of Staurosporine. Reagents for the fluorescence polarization assay were added to Griener 384 half well plates at the following final concentrations in buffer (20mM HEPES (pH 7.4), 5mM $MgCl_2$, 2mM $MnCl_2$): tracer (1X PanVera tyrosine kinase green kit Cat. # P2836); antibody (1X PanVera tyrosine kinase green kit Cat. # P2836); EGF receptor kinase, 50 nM (PanVera Cat. # P2628); poly GT, 300 nM (Sigma Cat. # P-0275; ATP, 10 μM (Sigma Cat. # A-7699) in a final volume of 20 μL. All reagents were added with a Beckman Biomek 2000 automated pipetting instrument, plates were incubated for 90 minutes at room temperature, and fluorescence polarization was measured in a Tecan Ultra plate reader.

Fluorescence polarization technology is well suited to high-throughput screening since inhibition of macromolecular binding can be done rapidly, in solution, without any washing steps [73-75]. The availability of adequate supplies of well-characterized probe and target protein molecules is fundamental to development of HTS assays. Custom made, synthetic peptide or oligonucleotide probes labeled with fluorescein or other fluorescent dye molecule can be obtained commercially. Molecular target proteins produced by recombinant DNA technology, can be characterized by LC/MS to assure purity and identity of the reagents.

Commercial reagents are available for conduct of both tyrosine and serine/threonine kinase inhibition assays in a homogeneous mode using FP detection. The phosphorylation of proteins by protein kinases is critical to the regulation of many biological processes and defects in these pathways have been observed in many human diseases including cancer. Traditional kinase assays are time consuming and frequently require radioisotopes precluding automation and high throughput capacity. Fluorescence polarization (FP) assays are based on the principle that a fluorescently labeled compound, when excited by plane-polarized light, will emit polarized fluorescent light. The emitted polarization will differ from the excitation polarization because the fluorescent molecule is in motion during the time that elapses between excitation and emission. FP can be used to monitor molecular interactions in a homogeneous environment at equilibrium. The binding of a fluorescently labeled small molecule to a larger molecule can be assayed by a change in the rate of rotation of the fluorescent molecule. If, during excitation by plane-polarized light, molecules remain stationary, the emitted light will remain highly polarized. However, if the excited molecule rotates during excitation, the emitted light will be depolarized [76]. Since FP is a measure of this rotation rate, it is related to molecular volume of the fluorescent molecule or combination of bound molecules. FP can be utilized to measure the phosphorylation of a kinase substrate or the inhibition of this phosphorylation event. In this FP assay fluorescein-labeled phosphopeptide (tracer) and any unlabeled phosphoproteins or phosphopeptides that are produced during a kinase reaction will compete for binding to antiphosphopeptide antibodies (anti-p). In the absence of kinase activity, most of the tracer will bind to the anti-p resulting in a high polarization value as measured in a FP plate reader in mP (polarization) units. In the presence of kinase activity, anti-p will bind to both the reaction products and the tracer decreasing the amount of bound tracer and concomitantly decreasing the polarization of the sample. Thus, the change in FP is directly related to the amount of kinase activity. Inhibitors of specific kinases can be assayed by quantitating the changes in polarization when compounds are added to the kinase reaction mixture [77]. We have developed a low volume 384-well plate assay to screen for inhibitors of tyrosine kinases. Figure **3** shows some of the steps in the systematic optimization of the assay components for the EGF receptor kinase. Substrate (poly GT), ATP, enzyme, tracer, and antibody concentrations must all be titrated to determine the optimal concentrations. The dynamic range of the assay is determined by the difference between the fluorescence polarization values obtained for fluorescein labeled tracer alone (minus antibody) and the maximum value obtained for the entire reaction mixture without ATP under which conditions the enzyme does not function (minus ATP). Optimization experiments are necessary to determine a satisfactory dynamic range for the particular kinase being studied. Each of the experimental variables must be titrated to determine optimal concentration ranges for the enzyme reaction. In practice, one variable is tested at multiple concentrations while holding the concentration of the other variables constant. Figure **3A** illustrates that over a concentration range of 30-600 nM, the substrate concentration had little effect on the assay. Likewise, as indicated in Figure **3B**, a broad range of ATP concentrations supported kinase activity, with near maximal effect at a concentration of 10 nM. Dramatic effects were observed when the enzyme itself was titred. As illustrated in Figure **3C**, maximal activity in short-term incubations required 100 nM enzyme concentration. However, concentrations as low as 18 nM showed near maximal activity when reactions were allowed to proceed for extended times (up to 190 minutes). Results such as these permit selection of conditions which maximize dynamic range and Z' statistics while conserving expensive reagents and optimizing time considerations. Figure **3D** illustrates the effect of staurosporine as a kinase inhibitor in the optimized assay.

Conventional modes of fluorescence detection can be very useful for HTS assays. However, application of fluorescence detection in assays utilizing crude natural product extracts can be problematic, due to the high degree of intrinsic fluorescence of many kinds of extracts, especially plant extracts. Use of Europium-labeled reagents and time-resolved fluorescence detection, provides one approach to dealing with this problem [78].

CELL-BASED HTS ASSAYS

Molecular-targeted screening can be approached with a large variety of technologies. Assays may be based on analysis of whole-culture "cytoblots" [79,80] a novel technology which can potentially address a large number of targets, evaluation of compound effects on fluorescent or luminescent reporter constructs [79], or on functional effects on complex signaling pathways [81].

Cell-based assays can be utilized to assay drug effects on generalized pathways or more specific targets. Utilizing commercially available reagents (Homogeneous Caspases Assay, Roche Molecular Biochemicals, Indianapolis, IN) we have implemented a high-throughput, homogeneous screening assay for the detection of apoptosis applicable to a variety of human tumor cell lines. Activated caspase activity (aggregate activity of cellular caspases) is assessed as liberation of free rhodamine following incubation of treated cells with rhodamine-labeled (quenched) substrate solution. Unquenched rhodamine fluorescence is measured on a plate reader. Figure **4** shows the results of HTS using CEM (Figure **4A**) and HL-60 (Figure **4B**) human tumor cells with the 220 compounds of the NCI "Training Set" of

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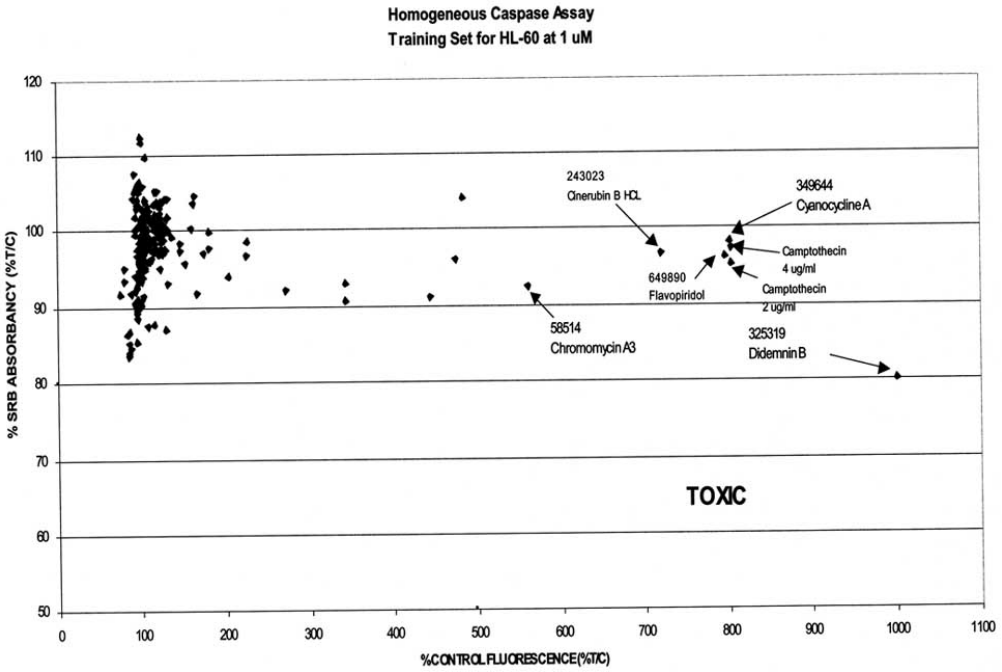

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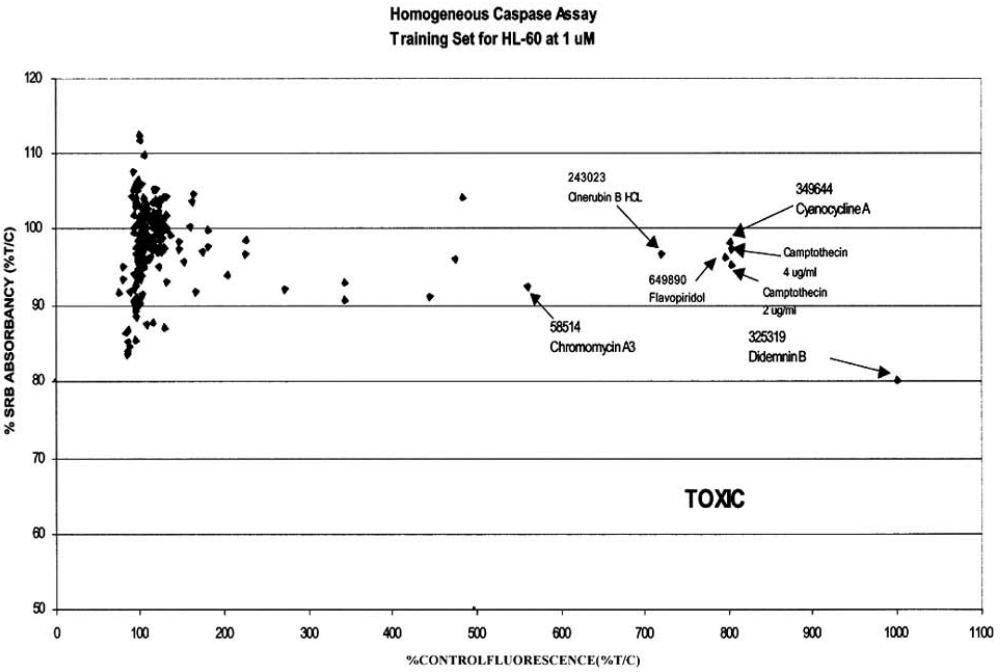


Fig. (4). HTS data from a generalized assay for induction of cellular caspase activity. HL-60 (20,000 cells/well) (Figure **4A**) and CEM (30,000 cells/well) (Figure **4B**) cells were inoculated using a Biomek 2000 automated pipetting instrument into 384-well black clear bottom plates, incubated for 24 hr. at 37°C in phenol-free RPMI 1640 medium supplemented with 5 % fetal bovine serum. Experimental compounds were then added with the Biomek 2000 and incubation was continued for 4 hr. at 37 °C. Substrate solution (Roche Cat. No. 3005372) was then added and after 1 hr. incubation at 37 °, fluorescence was measured in a Tecan Ultra plate reader with excitation at 485 nm and emission at 535 nm.

compounds at 1 uM concentration. Parallel assay plates were assayed for cytotoxicity using a SRB assay [9]. This latter assay was performed to enable the two-dimensional representation of assay results and establishment of activity criteria as shown in the figures. In the figures, compounds producing more than 80% growth inhibition were defined as "toxic". Compounds producing a five fold or greaterincrease in fluorescence intensity were defined as "active" inducers of caspase activity. A true high-throughput screening campaign would evaluate cytotoxicity in secondary testing. Under the conditions of this assay, the vast majority of Training Set compounds were found to be inactive and non-toxic. Camptothecin (NSC 94600) at 2 and 4 uM, serves as the positive control for this assay. Note that HL-60 cells appear somewhat more sensitive to induction of apoptosis, with five active compounds, in addition to the positive control treatments, compared to three active compounds in the CEM screen. The lack of complete overlap among the actives may reflect differences in operation of apoptotic processes in the different cell types or may represent fluctuations associated with operation of this assay in high-throughput mode. Didemnin B (NSC 325319) was most notably active in induction of caspase activity in both cell lines.

Cellular reporter constructs have found wide application in basic cell and molecular biological research. Non-radioactive versions have largely supplanted the classic chloramphenicol acetyl transferase (CAT) assays done on TLC plates and other reporters such as green fluorescent protein or luciferase have grown in popularity. These latter assays can be very sensitive and convenient for HTS. We have focused on luciferase assays, in part to avoid the problem of sample fluorescence mentioned above in relation to crude natural product extracts.

The hypoxia inducible factor, HIF-1 alpha, modulates expression of genes involved in cellular responses to low oxygen concentrations and is a major mediator of tumor angiogenesis. As one approach to development of a HTS addressing this pathway, we have utilized stable cell construct presenting multiple copies of the hypoxia responsive element driving expression of luciferase [82]. When placed under conditions of low (1%) oxygen concentration, this reporter is activated, resulting in production of luciferase which can be detected using commercial substrate reagents in 384-well microculture plates. The protocol for this assay is illustrated in Figure **5**. With this relatively simple protocol, high-throughput screening feasible with off-the-shelf equipment and a small team of technicians. Working on a part-time basis, a team of three technicians currently processes approximately 12,000 samples per month in this assay. Initial results of screening the NCI chemical library (at 1 uM) demonstrate that inhibitors of this pathway are detectible, with low frequency, and that some are capable of inhibiting down-stream processes such as VEGF secretion [83]. A full description of these results will be published elsewhere.

Another luciferase-reporter-based screen addresses the CEBP alpha transcription factor which plays a key role in controlling differentiation of myeloid cells [84]. Experimental evidence suggests that activation of this factor may promote cellular differentiation and thus exert an anti-leukemic effect in certain types of disease [85]. Exposure of

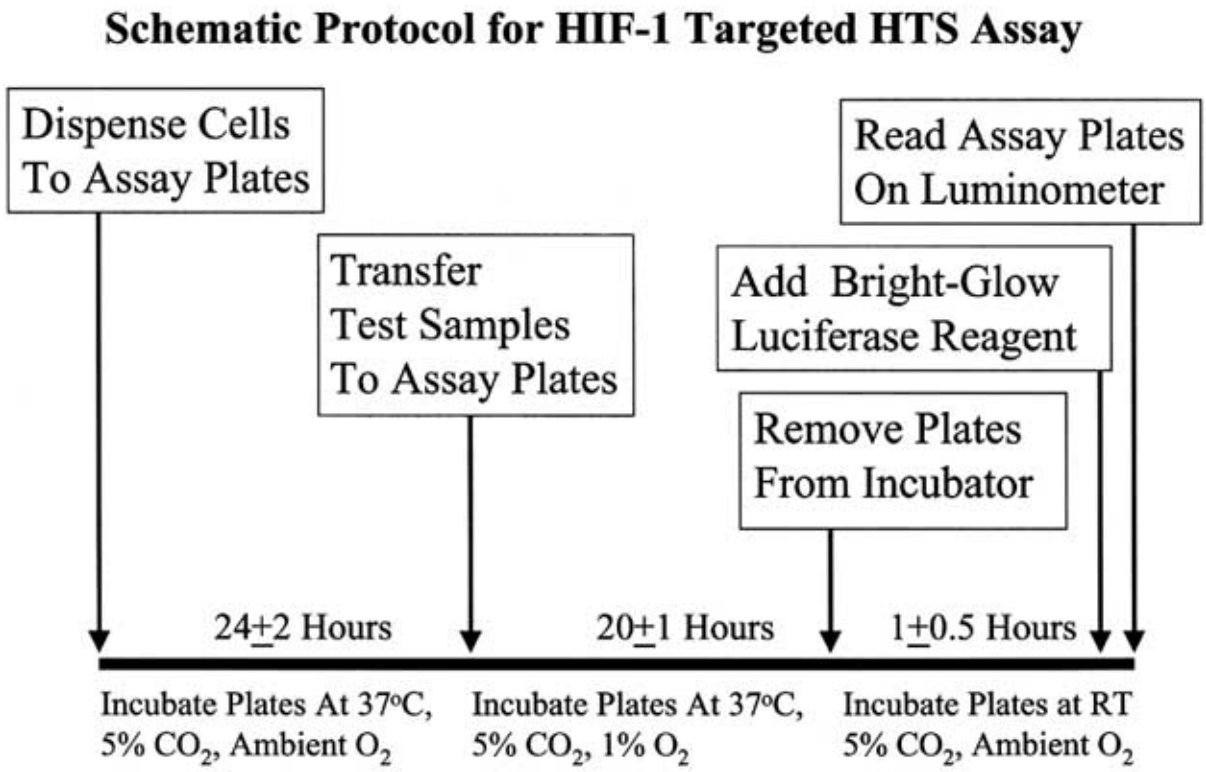


Fig. (5). Schematic protocol for HIF-1 alpha drug screening assay.

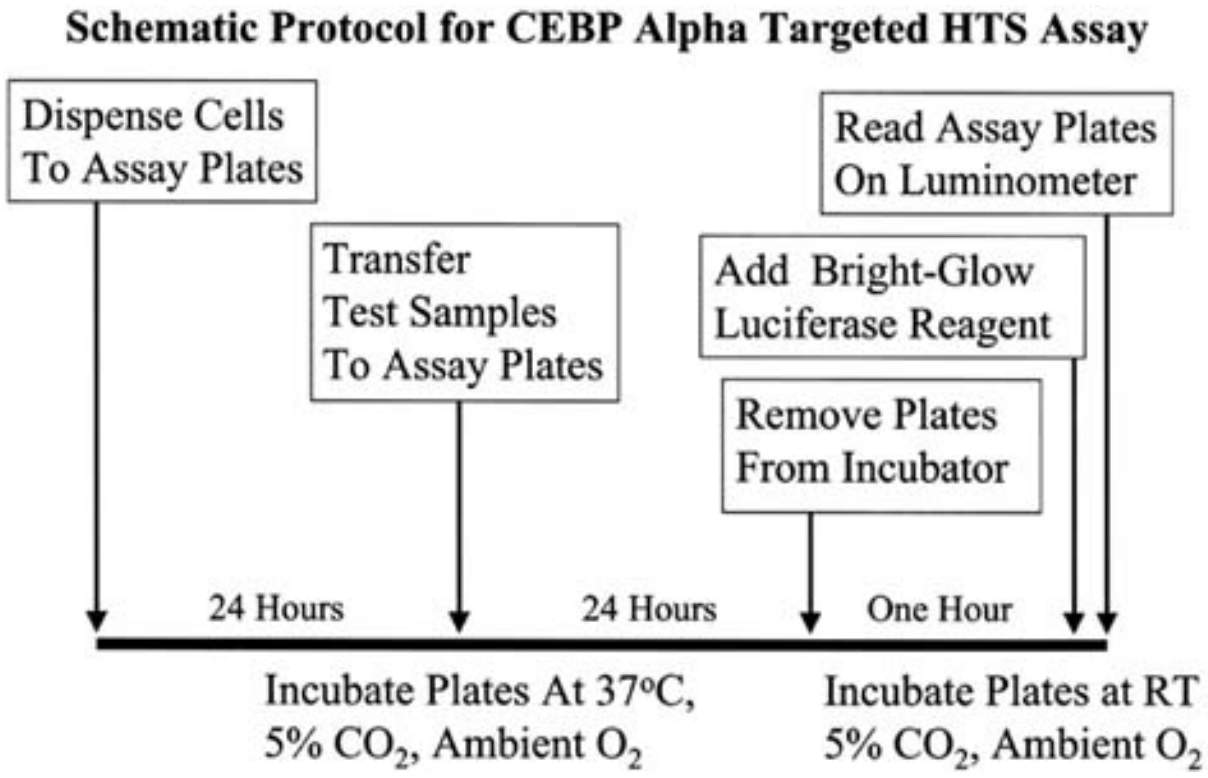


Fig. (6). Schematic protocol for CEBP alpha drug screening assay.

stably transfected U937 cells bearing a luciferase reporter driven by multiple copies of the CEPB alpha response element to retinoic acid results in activation of transcription. As described for the HIF-1 construct, this can be readily quantitated in assays performed in 384-well microculture plates. The protocol for this assay is illustrated in Figure **6**. This simple assay has even higher potential throughput than the HIF-1 assay, in that the manipulations required for low oxygen treatment are not required. Initial results of screening the NCI chemical library (at 1 uM) demonstrate that activators of this pathway are detectible, albeit with low frequency. Several activators have been identified which produce an activation effect dramatically greater than retinoic acid [86]. A full description of these results will be published elsewhere.

One of the first molecular targets addressed by DTP was the Met pathway. The met proto-oncogene receptor tyrosine kinase along with its ligand, hepatocyte growth factor/scatter factor (HGF/SF) has been implicated in tumor development and metastasis. Stimulation of HGF/SF results in the increase of urokinase (uPA) and it's receptor (uPAR) on the cell surface producing the proteolytic activation of plasminogen to plasmin initiating a cascade of proteolytic events leading to extracellular matrix degradation facilitating cell invasion [87]. We have developed a cell based high throughput 384-well plate assay to screen for inhibitors of the met signaling pathway. In this assay the conversion of plasminogen to plasmin is measured in MDCK (canine kidney) cells by the addition of chromozym* PL (Roche Molecular Biochemicals, IN) that is cleaved by plasmin to a chromogenic product quantitated on a microplate reader at 405 nm [88]. Figure **7** demonstrates the assay timeline for our high throughput met screen while Figure **8** illustrates the principle of the assay and the multiple potential points of interaction in the pathway. The anisamycin antibiotic, geldanamycin (NSC 122750), demonstrated to be an effective inhibitor of this pathway [88], serves as a positive control in this assay. Figure **9A** and **9B** show the results of the high throughput met screen for the 1,990 compounds of the NCI Diversity Set tested at 1 and 50 μM respectively. Data are presented in the same fashion as in Figure **4**, with a toxicity assessment on the X axis and the activity parameter on the Y axis. As before, the toxicity assessment was included for assay developmental purposes and would not normally be included in a primary high-throughput screening campaign. At both concentrations the vast majority of compounds were found to be inactive (no significant inhibition of the Met pathway) and non-toxic. Clearly, a larger portion of compounds was toxic at 50 μM (Figure **9B**) than at 1μM (Figure **9A**). Likewise, the yield of "active" compounds was higher at 50 μM (41 compounds) compared to 1μM (16 compounds). This latter yield represents a 0.8 % "hit" rate which would be quite manageable in a high-throughput screening campaign.

OPERATION OF HTS SCREENING MODELS

Typically, HTS involves screening an entire chemical library at a single concentration. This concentration can vary,

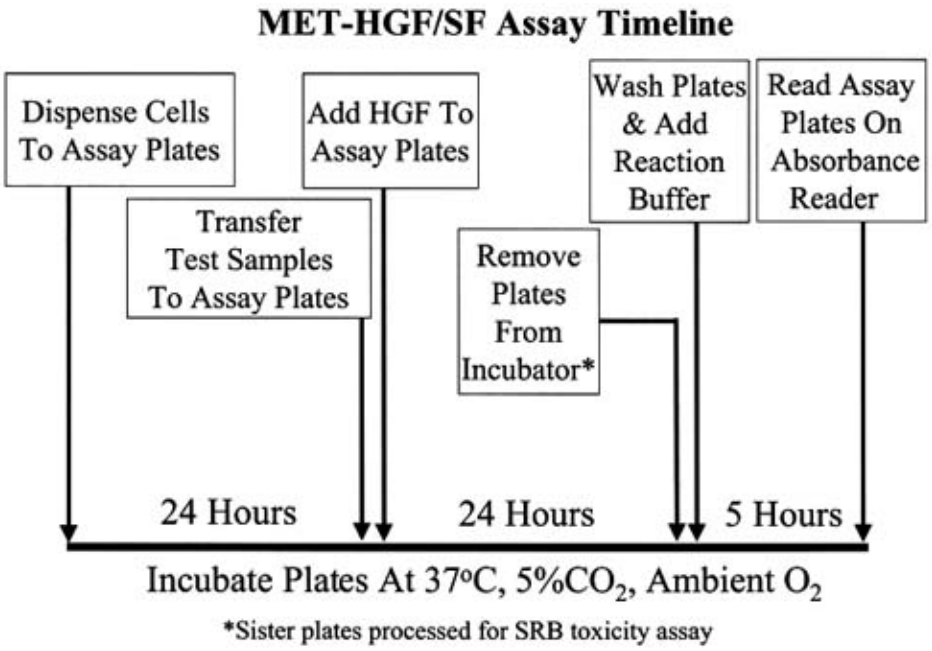


Fig. (7). Schematic protocol for Met drug screening assay.

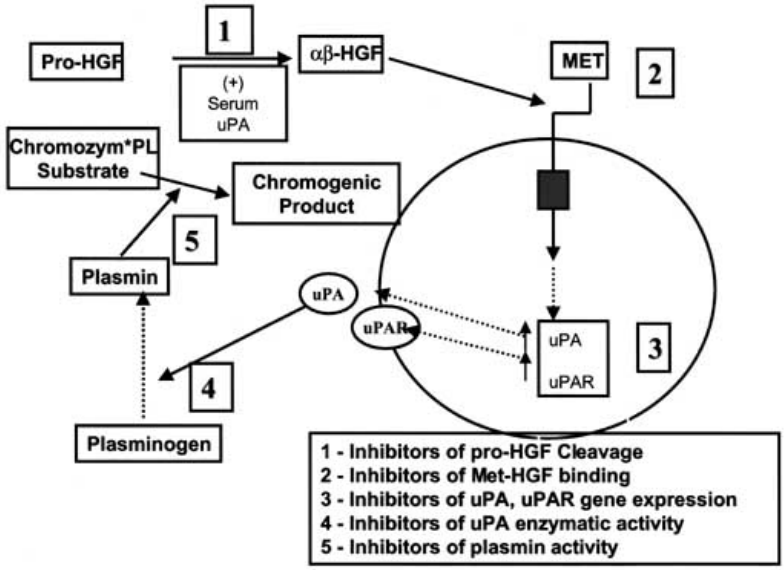


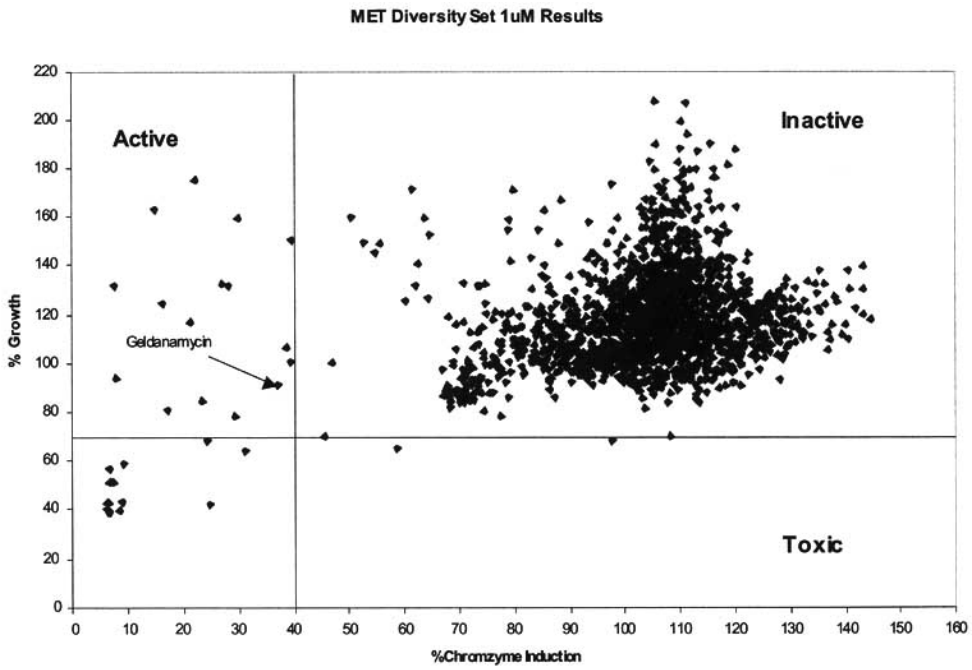
Fig. (8). Assay principle and multiple points of interaction in the screen for inhibitors of the Met signaling pathway. Five potential areas where pathway inhibition could occur are illustrated at: 1) pro-HGF cleavage to active form; 2) met-HGF binding; 3) uPA and uPAR gene transcription and expression; 4) uPA enzymatic activity and 5) plasmin activity.

depending on the performance characteristics of the assay and the nature of the library being screened. In general, screening concentrations are in the range of 0.1-100 μM for synthetic libraries and 1-250 μg/ml for natural product extracts. A generalized flow-chart for conduct of HTS campaigns is given in Figure **10**.

SECONDARY EVALUATION OF HTS LEADS

Secondary testing of "hits" from primary HTS usually involves confirmation of activity and assessment of potency of the effect. In some instances it may be convenient and efficient to obtain this information in one experiment by conducting a concentration-response assay that includes the concentration used for primary screening. Specificity of effect can be assessed by evaluating confirmed active samples in complementary assays addressing a different target. For cell-free assays this may involve testing in alternative models to identify compounds that non-specifically bind protein or nucleic acid targets or have a general effect in inhibiting enzymatic reactions. This latter endeavor can become quite complex when the target is the

A



B

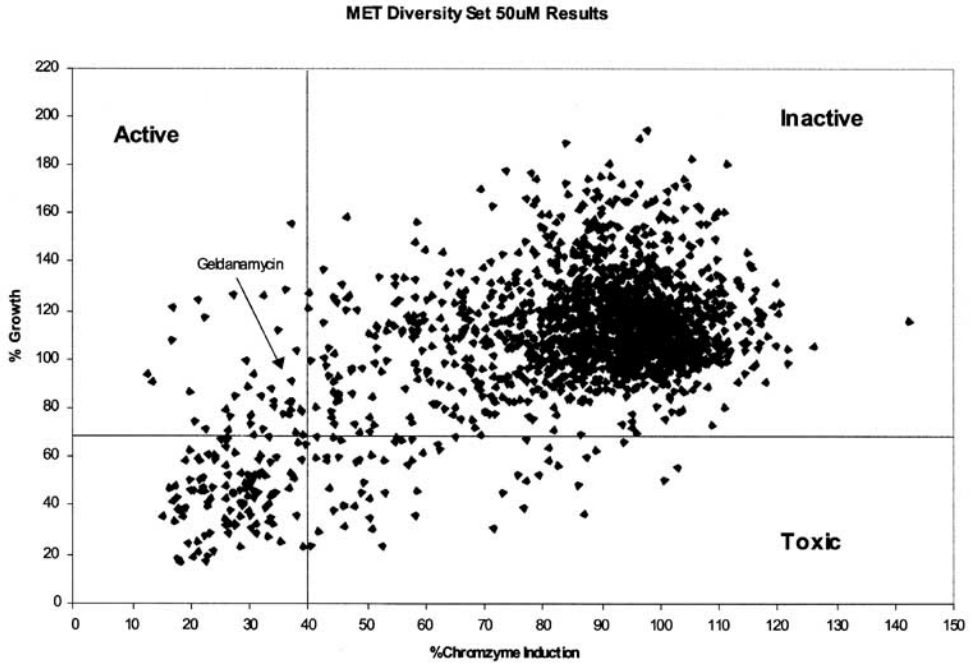


Fig. (9). Diversity Set results for the high-throughput Met assay at 1 μM (Figure **9A**) and 50μM (Figure **9B**). Madin-Darby Canine Kidney Cells (MDCK) cells, obtained from the American Type Culture Collection, were inoculated into flat bottom tissue culture 384 well plates using a Beckman Biomek 2000 automated pipetting instrument, incubated for 24 hr. at 37°C in DMEM medium supplemented with 10 % fetal bovine serum (FBS). After treatment with experimental compounds, HGF/SF (HGF) (R & D systems, Cat # 294-HG) was added to each well and cells were incubated for 24 hr. at 37°C. After incubation with HGF, cells were washed 2 times with DMEM without phenol red and FBS. Plasminogen and chromozym in Tris buffer were added to the plates with a Biomek 2000, and plates were incubated for 5 hr. at 37°C. Chromogenic product absorbency was measured in a Tecan Ultra plate reader at 405 nm. Duplicate plates were analyzed using the SRB assay to measure compound toxicity.

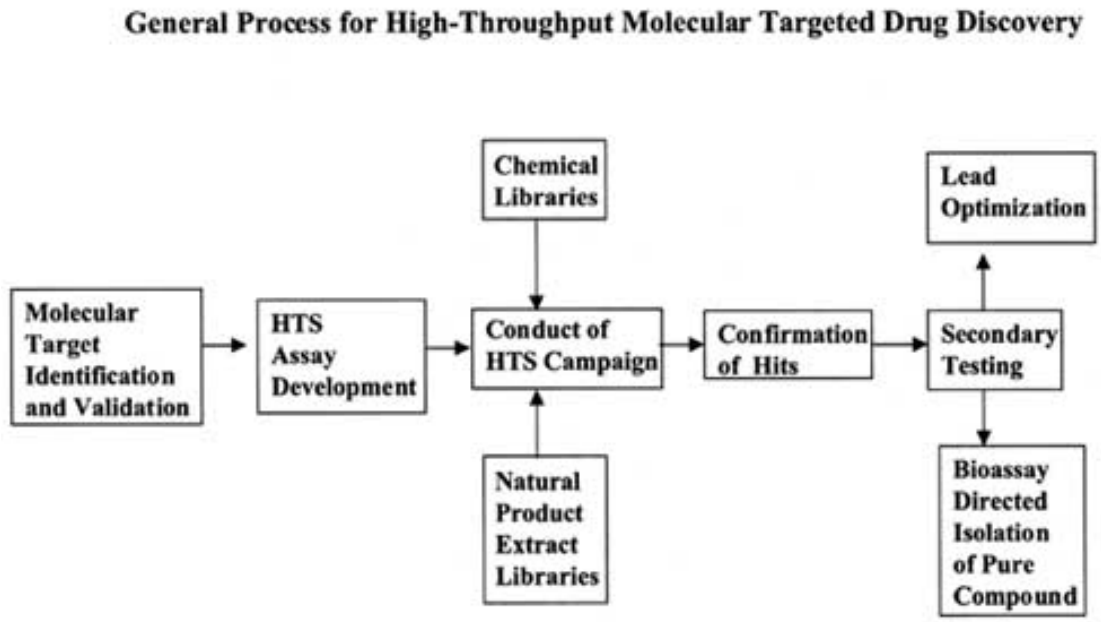


Fig. (10). A generalized flow-chart for conduct of HTS campaigns.

product of a large gene family or acts to perform a common function such as kinases or phosphatases. In the latter cases, rather extensive selectivity profiling may be necessary. Leads from cell-based assays can be initially tested in models presenting contrasting or mutated targets, to identify compounds active by virtue of an effect on the detection system or other artefactual reason. Because of the potential for action at many levels, leads from cell-based assays may require extensive testing to more fully define the mechanism by which a given molecule affects the target.

LEAD OPTIMIZATION

The goal of molecular-targeted HTS is to identify novel lead molecules with a reasonably potent effect on the target molecule or pathway. It is not expected to directly produce development candidates. Lead optimization is usually directed towards identifying more potent analogs with suitable pharmaceutical properties and can be addressed through testing of additional compounds selected from chemical libraries or generated by synthesis of selected compounds or generation of many analogs through parallel or combinatorial synthesis [89,90]. Using HTS assays for initial lead optimization makes it feasible to screen large numbers of analogs rapidly, in concentration-response fashion.

Natural product drug leads frequently present large, complex structures which represent major challenges for synthesis. Optimization of such molecules can be approached through synthetic modification, yielding semi-synthetic molecules such as 17-amino-geldanamycin. Alternatively, novel technologies for biological modification, using either enzymes derived from microbial organisms, or the organisms themselves, can generate libraries of derivatives for testing [91]. Members of such libraries can be re-generated using defined procedures potentially amenable to large-scale production.

Other pharmaceutical properties (ADME) must also be addressed in the lead optimization process. This topic has been the focus of several recent publications [92,93] and is the subject of considerable research. *In vitro* models can address potential for mutagenicity, metabolism, species-specific myelotoxicity, oral bioavailability, among other properties of potential lead molecules. This latter property is of greatly increased importance for molecular-targeted therapy which may need to be provided over an extended period of time.

ANIMAL MODELS

Pharmacokinetic testing as well as pharmacodynamic evaluation requires use of animal models. While a detailed discussion of this area is beyond the scope of this review, it is clear that a role exists for both conventional models and novel, specialized ones which present critical features of the molecular target of interest. Conventional models can support pharmacokinetic studies and play an important role in defining properties such as oral bioavailability. Tumor xenograft models may provide a useful context for efficacy testing in situations where the target molecule is known or expected to be present. The hollow-fiber model, in which tumor cell populations are loaded into biologically compatible microfibers which are subsequently implanted into animals [94], provides a physical means of recovering tumor cells following *in vivo* administration of drug which can be useful for development and validation of pharmacodynamic studies. Transgenic mice may provide a model which can present the target in the context of the role of the target in the natural history of particular malignancies [95-97]. Practical application of such models must address

issues related to production of adequate numbers of mice for experimental studies, staging of animals, scheduling of treatment, and statistical evaluation of therapeutic studies.

CONCLUSION

The technology for application of molecular-targeted, high-throughput drug screening is becoming widely available. Both large and small pharmaceutical companies are pursuing discovery efforts directed towards a large number of targets. Academic investigators have historically played a major role in identifying novel molecular targets and are becoming increasingly involved in efforts to exploit these targets for drug discovery. Such efforts are supported by several specialized grant mechanisms offered by the NCI (http://dtp.nci.nih.gov). As described above, the Developmental Therapeutics Program of the NCI has established facilities for conduct of HTS which are accessible to the extramural research community through a peer-reviewed process. Application of this technology by a diverse set of investigators addressing many targets and utilizing a variety of chemical libraries offers great potential for discovery of novel molecular targeted therapeutics in the next decade.

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