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ABSTRACT

Fluorescence polarization (FP) has been used to develop high throughput screening (HTS) assays for nuclear receptor-ligand displacement and kinase inhibition. FP is a solution-based, homogeneous technique requiring no immobilization or separation of reaction components. The FP-based estrogen receptor (ER) assay is based on the competition of fluorescein-labeled estradiol and estrogen-like compounds for binding to ER. These studies determined the K_d for this interaction to be 3 nM for ERα and 2 nM for ERβ; IC_{50} values for 17β-estradiol, tamoxifen, 4-OH-tamoxifen, and diethylstibestrol were determined to be 5.6, 189, 26, and 3.5 nM, respectively. In a screen of 50 lead compounds from a transcriptional activation screen, 21 compounds had IC_{50} values below 10 μM, with one having an almost 100-fold higher affinity for ERβ over ERα. These data show that an FP-based competitive binding assay can be used to screen diverse compounds with a broad range of binding affinities for ERs. The FP-based protein-tyrosine kinase (PTK) assay uses fluorescein-labeled phosphopeptides bound to anti-phosphotyrosine antibodies. Phosphopeptides generated by a kinase compete for this binding. In c-Src kinase reactions, polarization decreased with time as reaction products displaced the fluorescein-labeled phosphopeptide from the anti-phosphotyrosine antibodies. The experimentally determined IC_{50} of AG 1478 was 400 pM, while Genistein did not inhibit the epidermal growth factor receptor at similar concentrations. Like the FP-based PTK assay, the protein kinase C (PKC) assay utilizes competition. PKC isoforms had different turnover rates for the peptide substrate. The IC_{50} for staurosporine was less than 10 nM for all PKC isoforms. Tyrosine phosphatase assays use direct binding rather than competition. Increasing concentrations of T-cell protein–tyrosine phosphatase (TC PTP) increased the rate of dephosphorylation. This change in polarization was dependent on TC PTP and was inhibited by 50 μM Na_3VO_4. The IC_{50} of Na_3VO_4 was 4 nM for TC PTP. These data demonstrate that a FP-based assay can detect kinase and phosphatase activity. Homogeneous, fluorescent techniques such as FP are now methods of choice for screening many types of drug targets. New HTS instrumentation and assay methods like these make FP a technology easily incorporated into HTS.

INTRODUCTION

THE TREMENDOUS CHALLENGE facing the high throughput screening (HTS) scientist today is to screen more compounds against more targets using more quantitative and robust methods, without spending more money. Genomics efforts have flooded drug discovery with potential new drug targets.[1] New parallel combinatorial synthesis methods are providing more compounds that in turn must be screened for activity.[2] The process for identifying new lead compounds has to become more efficient. In order to increase the efficiency of HTS, new screening methods must be faster, cheaper, and more quantitative. Assays need to be miniaturized to decrease reagent costs and consumption of compound libraries. New assay formats must be homogeneous, requiring no separation of reaction components. Fluorescence methods are rapidly becoming the primary detection format in HTS[3] because they now approach the sensitivity of radioactive methods and are amenable to homogeneous, miniaturized formats. This report focuses on fluorescence polarization (FP) and how this technique is being used to improve HTS assays today.

PanVera Corporation, Madison, WI.
*Patent pending, PanVera Corporation.

Theory of fluorescence polarization

FP is used to study molecular interactions by monitoring changes in the apparent size of fluorescently labeled or inherently fluorescent molecules.[4–6] When a small fluorescent molecule (tracer) is excited with plane-polarized light, the emitted light is largely depolarized because the molecule rotates rapidly in solution during the fluorescence lifetime (the time between excitation and emission). However, if the tracer is bound to a larger receptor, thereby increasing its effective molecular volume, its rotation is slowed sufficiently to emit light in the same plane in which it was excited. The bound and free states of the tracer each have an intrinsic FP value, a high value for the bound state and a low value for the free state. The measured polarization is a weighted average of the two values, thus providing a direct measure of the fraction of tracer bound to receptor. An increase in molecular volume resulting from receptor-ligand,[7] DNA-protein,[8,9] or peptide-protein[10] binding or a decrease in molecular volume resulting from dissociation or enzymatic degradation[11,12] can be followed by FP.

FP enables the researcher to view molecular binding events in solution. It is a truly homogeneous technique in that no separation of bound and free species is necessary. Methods requiring separation are not only more time consuming, but they disturb the reaction equilibrium and therefore prevent accurate qualification of binding. Other fluorescence techniques are homogeneous, such as fluorescence resonance energy transfer (FRET) and homogeneous time-resolved fluorescence[3] (HTRF®; Packard Instrument Company, Meriden, CT). However, these techniques require multiple labeling reactions instead of one as in FP. Also, because the readout for FRET and HTRF is fluorescence intensity, there is a higher probability for artifacts from quenching and exogenous fluorescence. FP is a ratiometric measurement and therefore less sensitive to these sources of interference.

Assay development using fluorescence polarization

FP is best suited to observe the binding of small fluorescent molecules (<10 kDa) to larger proteins. The smaller the fluorescent molecule, the larger the dynamic range of the assay. Fluorescent labeling of peptides and DNA oligos is straightforward and can even be performed during synthesis. Labeling other small molecules is sometimes more involved, often requiring multiple attempts using a range of chemistries at multiple sites on the molecule. FP differs from other binding assay formats in that it detects the fraction bound of the small, fluorescent molecule (tracer) instead of the large molecule (receptor). Thus it requires saturating the tracer with receptor instead of the inverse approach used in most binding experiments. For this reason, FP requires large amounts of relatively pure protein. Two assay formats that have been particularly well suited to FP are receptor-ligand–binding assays and enzyme assays where the product can be quantified by competitive immunoassay. This article describes the development of two such assays: a ligand displacement assay for estrogen receptors (ERs) α and β, and competitive immunoassays for protein-tyrosine kinases (PTKs) and protein kinase C (PKC) family members.

Ligand displacement assays for estrogen receptors

Estrogen receptors (ERα and ERβ) are transcription factors that regulate the expression of genes involved in tissue growth and differentiation in diverse target tissues, including reproductive, skeletal, cardiovascular, and mammary tissue.[13–16] Estrogen acts by binding to ER, resulting in the transcriptional control of a variety of genes in target cells. Pharmacologists are looking for new estrogens and antiestrogens to treat a host of disorders such as breast cancer, Alzheimer's disease, cardiovascular disease, osteoporosis, type II diabetes, and urinary incontinence.[17]

Historically, estrogen activity has been assessed using *in vivo* assays, including whole animal[18] or cell-based assays such as proliferation in a breast carcinoma cell line[19] and induction of reporter gene expression from an estrogen-response element transfected into yeast or mammalian cells.[20] These require extensive manipulations of animals or live cells and response times of several hours to days. The time and extensive labor required for the *in vivo* method preclude their use in HTS.

In contrast, molecular ligand-binding assays are faster, more precise, and less labor intensive than *in vivo* assays because they are performed *in vitro* with isolated components. Molecular assays are not limited by toxicity or cell permeability of the test compounds, factors that limit the type of compounds and concentrations that can be tested in a cell-based assay. To develop ligand-binding assays more suited to a HTS format, investigators have begun using various types of FlashPlate™ (NEN® Life Science Products, Boston, MA) or scintillation proximity assays (SPA, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Receptor and the scintillant are bound to a solid phase to ensure that only the receptor-bound labeled estrogen, and not the excess free ligand, is close enough to excite the scintillant.[21] These approaches require no separation of bound and free ligand, but still rely on the use of radioisotopes and immobilization of the receptor, which could cause disadvantageous conformational changes. These shortcomings prevent the widespread adaptation of current ligand-binding assays to HTS formats.

The FP-based ER assay is a homogeneous, fluorescent assay requiring no immobilization and is well suited for HTS. Fluorescein-labeled estradiol and estrogen-like compounds compete for binding to the estrogen receptor. When there are no competing compounds present, the fluorescein-labeled estradiol will be bound by the estrogen receptor, resulting in a high FP value. However, in the presence of estrogen-like competitors, the fluorescein-labeled estradiol is displaced from the estrogen receptor, resulting in a decreased FP value. Because the observed FP value is a weighted average of the FP values of the individual bound and free tracer and therefore is a direct measure of the fraction bound, polarization may be plotted against the logarithm of the compound concentration to determine the IC_{50} for the compound under the assay conditions tested. Figure 1 illustrates these principles.

Protein-tyrosine kinases assays

PTKs catalyze the transfer of a high-energy phosphoryl group from ATP to the hydroxyl group of a tyrosine, whereas tyrosine phosphatases cleave the phosphate from phosphotyrosine. Phosphorylation appears to be the "master" biochemical

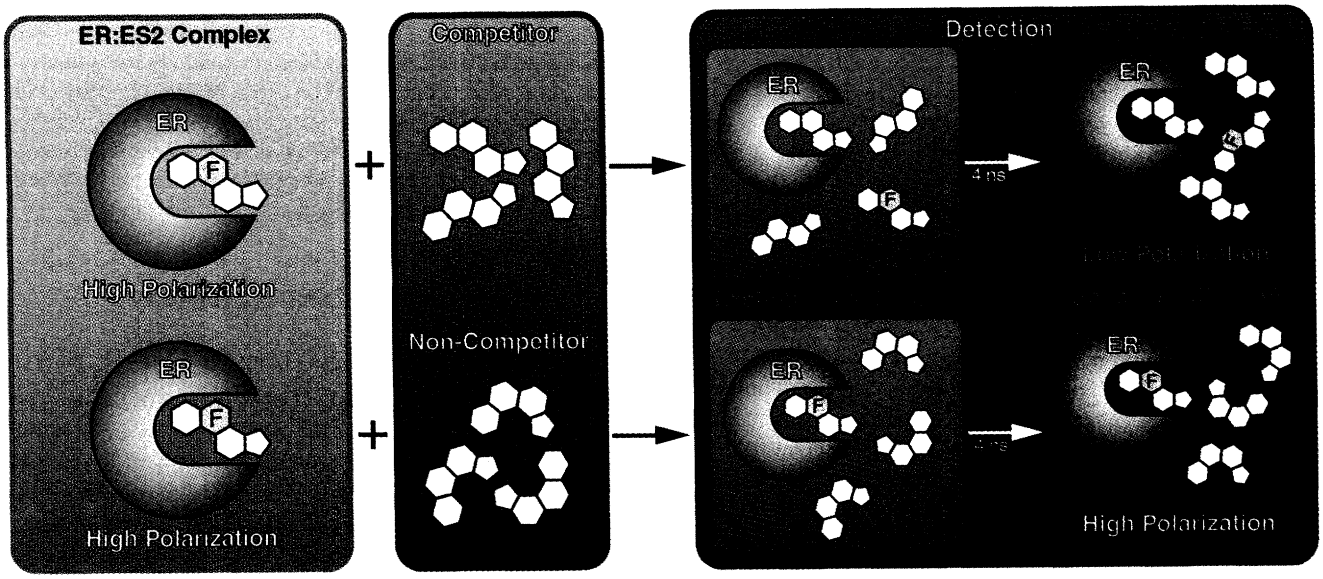

FIG. 1. In the ER competitor assays, an ER-ES2 complex is added to a test compound. Estrogen competitors displace ES2 from the ER-ES2 complex, causing ES2 to tumble rapidly during its 4-ns fluorescence lifetime, resulting in a reduced FP value. Noncompetitors will not displace the ES2 from the ER-ES2 complex, so the FP value remains high. The shift in polarization in the presence of test compounds is used to determine the relative affinity of test compounds for ERα or ERβ.

reaction in signal transduction, and it has been estimated that at least one third of the proteins in the average mammalian cell are phosphorylated.[22] The regulated and reversible phosphorylation of tyrosines is critical to the normal regulation of many biological mechanisms, including cell growth, proliferation, differentiation, motility, transcription, synaptic function, and metabolism.[22–24]

There have been more than 95 PTKs and 55 tyrosine phosphatase genes found in humans.[23] Defects in these signal transduction pathways can result in a number of human diseases, including cancer.[22,24] For example, in the case of the epidermal growth factor (EGF) receptor, overexpression and mutation have been associated with some of the most incurable cancers, including glial and pancreatic tumors.[26–27] The development of anticancer agents based on the inhibition of EGF binding to the receptor, however, has had limited success.[28] Efforts to screen for new tyrosine kinase inhibitors have been hindered by the lack of robust, high throughput tyrosine kinase assays.

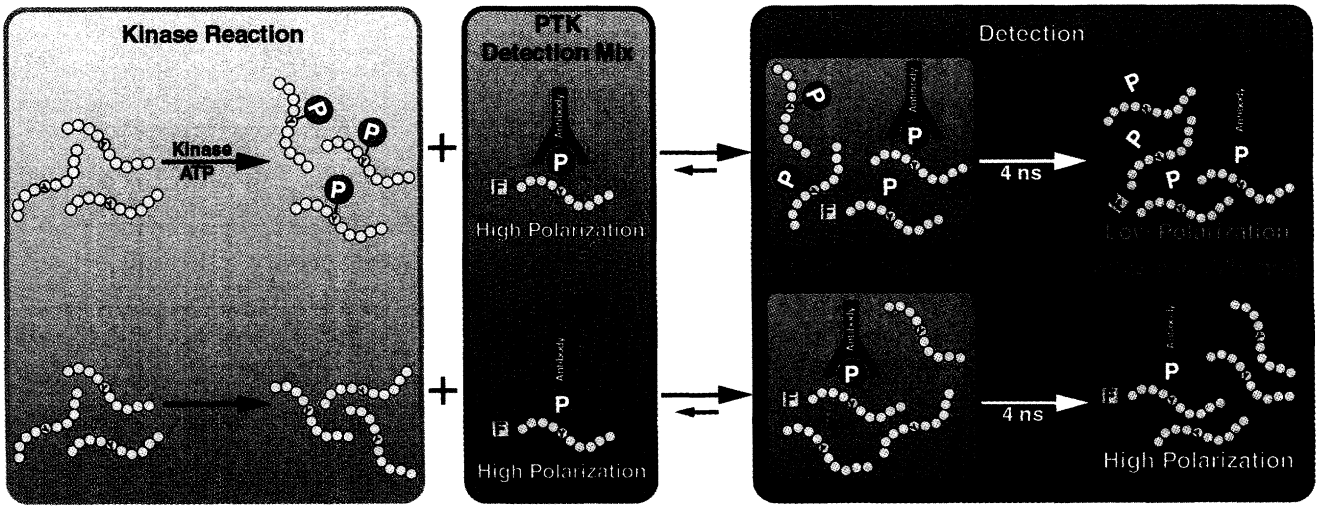

FIG. 2. This figure captures the general theme of the FP-based competitive immunoassay for PTK activity. In the top reaction, the addition of a tyrosine kinase and ATP leads to the phosphorylation (P) of the peptide substrate on a tyrosine residue (Y). After the reaction is quenched, the PTK Detection Mix, which has a high polarization and contains an anti-pY Ab (Antibody) bound to a fluorescein-labeled (F) phosphopeptide tracer, is added and the system reaches equilibrium. In the detection phase of the experiment, the tracer is displaced from the anti-pY Ab by kinase reaction products, causing the tracer to tumble rapidly during its 4 ns fluorescence lifetime, resulting in a low FP signal. When no kinase reaction products are present, the tracer-antibody complex remains intact, with no change in the high FP value.

Current tyrosine kinase assay formats include ELISA- and radioimmunoassay-based methods. These methods require immobilization of reaction components on plates, as well as multiple separation and washing steps, making them difficult to implement in HTS. Although radioactive methods are very sensitive, they create significant radioactive waste. Newer homogeneous methods now in use include SPA[29] and HTRF.[30] SPA is a radioactive technique requiring immobilization, and HTRF requires labeling and characterizing multiple assay components.

The FP-based PTK assay is a homogeneous, fluorescent assay requiring no immobilization that is well suited for HTS. A fluorescein-labeled phosphopeptide (tracer) and any unlabeled phosphopeptides generated during a kinase reaction compete for binding to anti-phosphotyrosine antibodies (anti-pY Ab). When there are no kinase reaction products present, the tracer will be bound by the anti-pY Ab, resulting in a high FP value. However, after a PTK reaction has occurred, reaction products displace the tracer from the anti-pY Ab, resulting in a decreased FP value. The observed FP value is a weighted average of the FP values of the individual bound and free tracer and therefore is a direct measure of the fraction bound. If sufficient competitor phosphopeptide is generated during the PTK reaction, the tracer will be totally displaced from the anti-pY Ab and the emitted light will be totally depolarized. Thus the change in FP value is directly related to the amount of the PTK activity. These principles are diagrammed in Figure 2. Measurements can be made in real time, allowing the observed enzymatic activity to be monitored both kinetically or in an endpoint assay format.

The general principles described here can also be applied to a tyrosine phosphatase assay, which, like the kinase assay, can be monitored as either a kinetic or an endpoint assay. Phosphatase assays are slightly different from the competition-based PTK assays described earlier in that reaction progress is monitored using direct binding rather than competition. Because the tracer is dephosphorylated by a phosphatase, it can no longer be bound by the anti-pY Ab, increasing the free fraction and decreasing the polarization of the sample.

Protein kinase C assay

The phosphorylation of serine and threonine residues in proteins by PKC family members is critical to the normal regulation of many biological mechanisms, including the modulation of membrane structure, receptor desensitization, transcriptional control, cell growth and differentiation, and the mediation of immune response. PKCs also play a role in memory, learning, other long-term functions, and various pathological processes.[31–33] A number of studies have suggested that inappropriate activation of PKCs can contribute to cancer, inflammation, viral infection, immune and central nervous system disorders, cardiovascular malfunction, vascular complications of diabetes, and insulin resistance.[34] Therefore, identifying PKC inhibitors is essential to developing new therapies for these diseases.

Like the FP-based PTK assay, the principle behind the PKC assay is competition. Fluorescein-labeled phosphopeptide tracers and any unlabeled phosphopeptide products generated during a PKC reaction will compete with each other for binding to anti-phosphoserine antibodies. In a reaction mixture containing no phosphopeptide products, a significant portion of the fluorescent tracer will be bound by the antibody, resulting in a high polarization value. However, in a reaction mixture containing phosphopeptide products, some of the tracer will be displaced from the antibody and the emission signal will become depolarized. Therefore, the change in polarization is directly related to the amount of PKC activity in a reaction.

MATERIALS AND METHODS

FP-based ligand displacement assay for ERα and ERβ

The results of direct binding studies of a fluorescent estradiol to ERα or ERβ using the FP method are presented in Figure 3. The assay consists of recombinant, human ERα or ERβ (PanVera Corporation, Madison, WI), and a fluorescein-labeled estradiol, Fluormone™ ES2 (PanVera Corporation). Using a 96-well plate (DYNEX, Chantilly, VA) and Estrogen Screening Buffer (PanVera Corporation) as the diluent, a constant amount of ES2 was titrated with increasing concentrations of ERα or ERβ. The FP value was measured at each receptor concentration on a Polarion instrument (TECAN, Research Triangle Park, NC) using 483-nM excitation and 536-nM emission filters. These data were then used to determine the equilibrium binding constants.

Displacement of bound ES2 by estrogen ligands. Four compounds, 17β-estradiol, tamoxifen, its active metabolite 4-OH-tamoxifen, and diethylstilbestrol (DES), were obtained and tested for their ability to displace ES2 from an ERα- or ERβ-

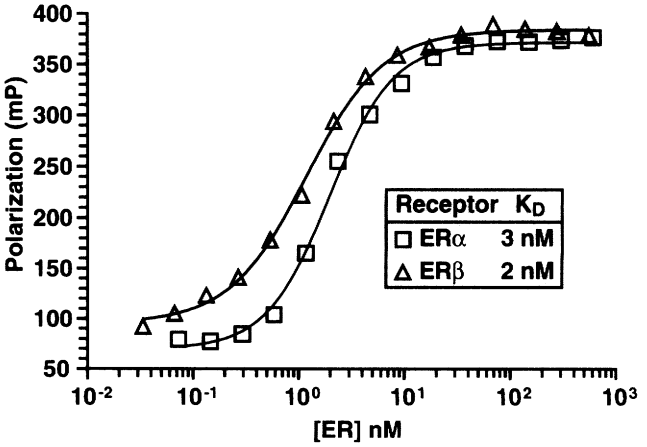

FIG. 3. A saturation-binding isotherm was generated in triplicate for ERα and ERβ binding to ES2. Both ERα and ERβ were diluted in buffer (100 mM potassium phosphate [pH 7.4] containing 100 μg/ml bovine gamma globulin [BGG]) to a final volume of 100 μl on a 96-well plate. ES2 was then added to each well to a final concentration of 1 nM. The plate was mixed and incubated at room temperature for 2 h. The polarization (mP, mean ± 1 standard deviation) was plotted against an increasing concentration of ER and the equilibrium binding constant (K_d) was calculated using nonlinear least squares analysis using Prism software. K_d values determined for ERα and ERβ were 3 nM and 2 nM, respectively.

ES2 complex. Because the large receptor-ligand complex tumbles slowly and has a high FP value, increasing concentrations of a competing ligand displace ES2 from ER. Free ES2 molecules are then able to tumble more rapidly and therefore have a low FP value. Because the measured polarization is the weighted average of the bound and free ES2 molecules, it can be directly used to calculate percent inhibition. All compounds were prepared as a 10 mM stock in DMSO (PanVera Corporation). In triplicate, tamoxifen (Sigma Chemical Co., St. Louis, MO), 4-OH-tamoxifen (Sigma), DES (Steraloids, Newport, RI), and 17β-estradiol (Sigma) were serially diluted in bovine gamma globulin/phosphate buffer (PanVera Corporation) on a 96-well plate (DYNEX). A mixture of either ERα or ERβ (PanVera Corporation) and Fluormone™ ES2 (PanVera Corporation) was added to the diluted inhibitors. The final concentration of ES2 in all wells was 1 nM. For ERα experiments, the final concentration of the receptor was 13 nM, while in ERβ experiments, the receptor was at a final concentration of 10 nM. The plate was then incubated at room temperature for 2 h. The polarization was measured on a TECAN Polarion using the recommended fluorescein filter set. The polarization (in millipolarization [mP]) was plotted against increasing concentrations of the test compounds. IC_{50} values were calculated using Prism® (GraphPad Software, Inc., San Diego, CA), a nonlinear least squares curve-fitting program.

ER competitor assays for HTS. As a feasibility study (and using techniques and reagents identical to those in the previous section), we tested 43 of our pharmaceutical collaborator's lead compounds from an ER-responsive gene reporter assay. Each plate tested contained several controls, including 17β-estradiol and DES. Our aim was to identify compounds selective for either ERα or ERβ.

Protein-tyrosine kinase assay

Reagent development for the FP-based PTK assay. The two critical components in the FP PTK assay are a fluorescent phosphopeptide tracer and an anti-pY Ab. In general, the antibody should bind the tracer with high affinity and exhibit a large shift in polarization between the bound and free tracer. High-affinity anti-pY antibodies enable use of lower concentrations in an assay. This is critical for both assay sensitivity and cost containment, considering both the cost of commercially available antibodies and the cost-per-well demands of HTS groups. Also, the anti-pY Ab used must be able to bind to both the tracer and phosphopeptide reaction products with similar affinity. For these assays, the tracer was synthesized by Genosys (The Woodlands, TX) and was optimized for maximum polarization shift, which included changing the sequence and length of the peptide, which was derived from a naturally occurring source. Further modifications could have included altering the fluorophore position or coupling chemistry. The goal was to maximize the dynamic range of the assay (polarization shift from bound to free tracer) without sacrificing affinity.

Kinetic and endpoint PTK assays. The PTK kinase assay was used to measure kinase activity of c-Src kinase (Signase, Houston, TX) in an endpoint mode. In a 96-well plate (DYNEX), parallel kinase reactions were run at room temperature for a maximum of 10 min in a final volume of 40 μl under the following conditions: 50 mM HEPES (pH 7.4), 6 mM $MgCl_2$, 0.1 ng/μl c-Src, 1.25 mM ATP, and 0.125 mg/μl poly(Glu, Tyr) 4:1 substrate (Sigma). Just after the addition of ATP to start the reaction and every 2 min after that, 10 μl of 100 mM EDTA was added to quench the reaction. After all of the reactions were quenched, 50 μl of the PTK Detection Mix (PanVera Corporation), which contains the F-phosphopeptide tracer–anti-pY Ab complex, was added to each well and the reaction was incubated for 5 min. The FP signal was then read at room temperature on a TECAN Polarion. Twenty hours later the polarization was read again to test the stability of a quenched PTK assay after the addition of the tracer–anti-pY Ab complex.

The activity of c-Src (Signase) was also assayed in the kinetic mode of the FP instrument by measuring the polarization signal in 1 min intervals over the duration of a 1-h experiment. In a 96-well plate (DYNEX), five separate kinase reactions were run at room temperature in a final volume of 100 μl. All reactions contained 50 μl of the PTK Detection Mix. The first three reactions contained either 1, 5, or 10 ng of c-Src (equal to 0.01, 0.05, and 0.1 ng/μl, respectively) with the following the reaction conditions: 50 mM HEPES (pH 7.4), 6 mM $MgCl_2$, 1.25 mM ATP, and 0.125 mg/μl poly(Glu, Tyr) 4:1 substrate (Sigma). Two control reactions were also run; one did not receive ATP, while the second did not receive the poly(Glu, Tyr) 4:1 substrate. ATP was added last to start each reaction.

Inhibition of EGF receptor by two compounds. By adding unknown compounds to the kinase reaction, inhibitors or activators can be screened and IC_{50} values established. Inhibition of the EGF receptor (PanVera Corporation) by two different compounds was examined. Compound AG 1478 has a published IC_{50} in the nanomolar range, while Genistein inhibits EGF receptor at micromolar concentrations. Both compounds were obtained from Calbiochem (San Diego, CA). In a black, round-bottom, 96-well plate (DYNEX), EGF receptor (PanVera Corporation) was preincubated with 2-fold serial dilutions of the inhibitors for 5 min; ATP was then added to start the reactions. Final conditions were 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 50 μM Na_3VO_4, 1 nM EGF receptor, 1 mM ATP, 1% DMSO, and 1 mg/ml poly(Glu, Tyr) 4:1 (Sigma) substrate. The reaction was run for 2 h at room temperature and quenched with 20 mM EDTA. An equal volume of the PTK Detection Mix (PanVera Corporation) was added and the reaction was incubated for 5 min. The results were read at room temperature on a TECAN Polarion, and nonlinear regression analysis was performed on a semi-log plot of the data to determine the IC_{50} of each compound.

Protein-tyrosine phosphatase essay

The enzymatic activity of T-cell protein-tyrosine phosphatase (TC PTP) was measured in a dose-dependent manner by incubating different concentrations of TC PTP (New England Biolabs, Beverly, MA), from 0.05 U/μl to 0.0005 U/μl, with 50 μl of the PTK Detection Mix (PanVera Corporation) in a final volume of 100 μl. Two control assays were performed: one did not receive TC PTP, while the other was supplemented with 50 μM Na_3VO_4 (Sigma), a potent phosphatase inhibitor.

Inhibition of TC PTP by sodium vanadate. The IC_{50} values for Na_3VO_4 and TC PTP were determined in the following manner. In a 96-well plate (DYNEX), Na_3VO_4 (Sigma) was serially diluted 2-fold into 24 wells from a starting concentration of 500 nM in a volume of 50 μl. TC PTP was then added to each well so that the final concentration of the enzyme would be 0.005 U/μl per assay. To start the reaction, 50 μl of the PTK Detection Mix (PanVera Corporation) was added, and each reaction was incubated for 15 min. At the end of the incubation, 10 μl of 550 μM Na_3VO_4 was added to quench all of the reactions, and the polarization for each sample was measured on a TECAN Polarion. Nonlinear regression analysis of a semi-log plot of the data was used to analyze these data.

Protein kinase C assay

Reagent development for the FP-based PKC assay. The PKC assay requires two main reagents and four general steps. The reagents are very similar to those of the PTK assay. A fluorescein-labeled phosphoserine-containing peptide and an antibody that recognizes this phosphopeptide are required. For this assay to work, the tracer must be competitively displaced from the antibody by unlabeled phosphopeptides or phosphoproteins.

When performing the assay, the kinase, lipids, calcium, magnesium, fluorescent tracer, peptide substrate, and potential PKC inhibitor are combined. Next, ATP is added to start the reaction. Following an incubation period, a quench/detection mixture containing EDTA and anti-phosphoserine antibody is added to stop the kinase reaction and initiate the competition for antibody binding. Finally, polarization values are measured to determine the extent of PKC activity in the reaction.

Measuring the enzymatic activity of seven PKC isoforms. Using this basic experimental procedure, a dilution series of seven PKC isoforms (PanVera Corporation) were tested to determine the amount of each isoform required to achieve the necessary turnover in a 90-min reaction. The final amount of each PKC isoform ranged from 10 pg to 20 ng per well. After a 5-min incubation with additional PKC reaction components, ATP was added to initiate the reactions. Final reaction conditions (for a 50-μl total reaction volume) were 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 μM $CaCl_2$, 0.1 mg/ml phosphatidylserine, 0.02 mg/ml diacylglycerol, 2 μM peptide substrate, 2× PKC fluorescent phosphopeptide tracer, 50 μM sodium vanadate, and 10 μM ATP.

After a 90-min incubation at room temperature, 50 μl of a quench/detection mixture (containing 130 mM EDTA and anti-phosphoserine antibodies) was added to each well to stop the reaction and initiate the competition for antibody binding. Following an additional 30-min incubation at room temperature, the FP of each well was measured on a TECAN Polarion.

Inhibition of PKC isoforms by staurosporine. Staurosporine IC_{50} values were determined for six PKC isoforms using the amount of each isoform required for sufficient reaction turnover. In a round-bottom, 96-well plate, PKC isoforms (15 ng PKCα, 300 ng PKCβI, 300 ng PKCβII, 17 ng PKCγ, 117 ng PKCδ, and 65 ng PKCϵ) were individually incubated with serial dilutions of staurosporine (Calbiochem) for 5 min at room temperature. ATP was then added to start the reactions. Final reaction conditions (for 50-μl total reaction volumes) were 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 μM $CaCl_2$, 0.1 mg/ml phosphatidylserine, 0.02 mg/ml diacylglycerol, 2 μM peptide substrate, 2× PKC fluorescent phosphopeptide tracer, 50 μM sodium vanadate, 0.02% NP40, and 10 μM ATP. After a 90-min incubation at room temperature, 50 μl of a quench/detection mixture (containing 130 mM EDTA and the anti-phosphoserine antibody) was added to stop each reaction and initiate the competition for antibody binding. Results were measured on a TECAN Polarion, and nonlinear regression analysis was performed on a semi-log plot of the data.

RESULTS

Estrogen receptor assays

The FP value was measured at each receptor concentration and was used to calculate the equilibrium binding constants for each receptor. Using the log of the receptor concentration and polarization (mP), nonlinear regression analysis determined the K_d to be 3 nM for ERα and 2 nM for ERβ (Fig. 3).

IC_{50} values for 17β-estradiol, tamoxifen, 4-OH-tamoxifen, and DES, calculated using a nonlinear least squares curve-fit-

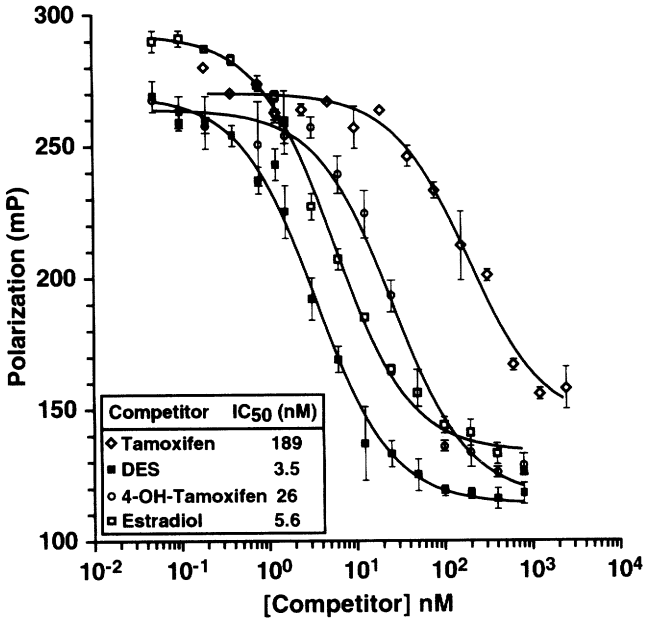

FIG. 4. The affinities of a panel of known estrogen ligands for ERβ were determined by their ability to displace the fluorescent ligand ES2 from ERβ using the FP-based competition assay. All compounds were prepared as a 10 mM stock in DMSO. Tamoxifen, its active metabolite 4-OH-tamoxifen, DES, and 17β-estradiol were serially diluted in BGG/phosphate buffer on a 96-well plate. A mixture of either ERα or ERβ and ES2 was added to the diluted inhibitors to a final concentration of 13 nM for ERα, 10 nM for ERβ, and 1 nM for ES2, and incubated at room temperature for 2 h. The polarization was measured on a TECAN Polarion using the recommended fluorescein filter set. The polarization (mP) was plotted against the log of the test compound concentration with Prism software. The IC_{50} values were calculated using a nonlinear least squares curve-fitting program.

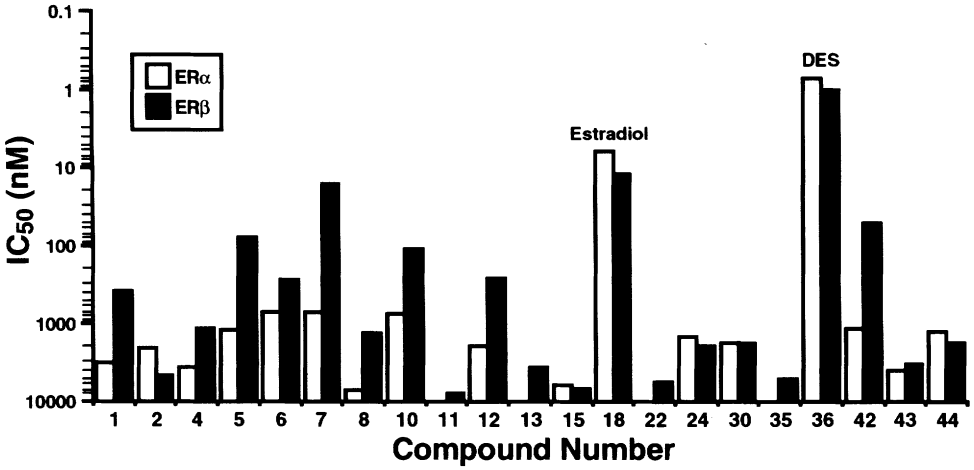

FIG. 5. Binding affinities of 43 lead compounds from an ER-responsive gene reporter assay and 7 controls were determined in a blind study using the FP assay. All compounds were prepared as a 10 mM stock in DMSO. The compounds were then serially diluted in BGG/phosphate buffer on a 96-well plate. A mixture of either ERα or ERβ and ES2 was added to the diluted inhibitors to a final concentration of 13 nM or ERα, 10 nM for ERβ, and 1 nM for ES2, and incubated at room temperature for 2 h; the polarization was then measured on a TECAN Polarion. The polarization (mP) was plotted against the log of the compound concentration with Prism software, and the IC_{50} values were calculated using a nonlinear least squares curve-fitting program. Of the original 50 compounds tested, those with IC_{50} values lower than 10 μM, including estradiol and DES, are plotted here. Higher bars represent higher affinity binding.

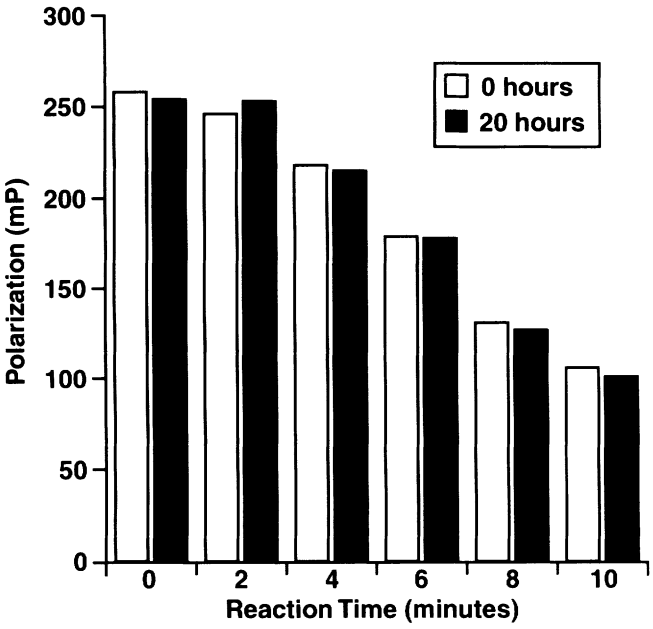

FIG. 6. The PTK activity of the tyrosine kinase c-Src was assayed by measuring the change in FP signal over time in an endpoint experiment. In a 96-well plate, parallel kinase reactions were run at room temperature for a maximum of 10 min in a final volume of 40 μl under the following conditions: 50 mM HEPES (pH 7.4), 6 mM $MgCl_2$, 0.1 ng/μl c-Src, 1.25 mM ATP, and 0.125 mg/μl poly(Glu, Tyr) 4:1 substrate. Just after the addition of ATP to start the reaction and every 2 min after that, 10 μl of 100 mM EDTA was added to quench the reaction. After all of the reactions were quenched, 50 μl of tracer: Ab complex, was added to each well and the reaction was incubated for 5 min. The FP signal was then read at room temperature on a TECAN Polarion. Twenty hours later, the polarization was read again to test the stability of a quenched PTK assay after the addition of the tracer–anti-pY Ab complex.

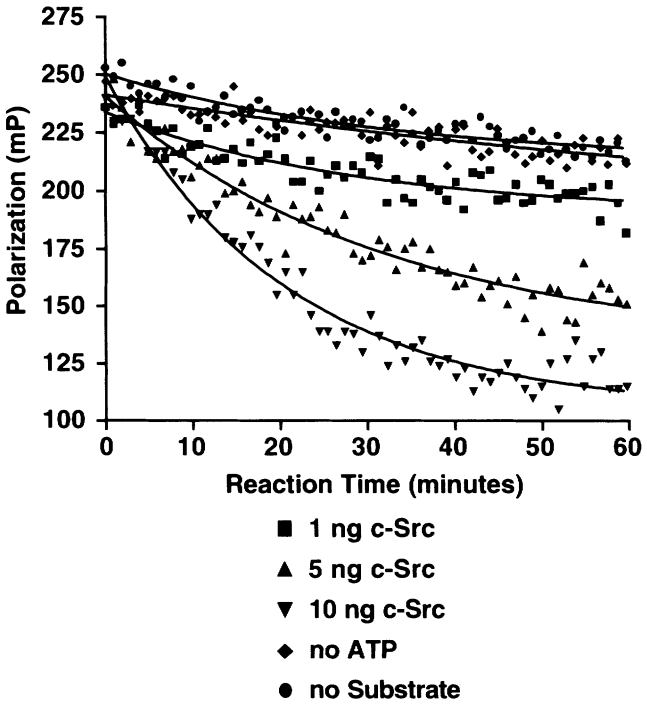

FIG. 7. The PTK activity of c-Src was also assayed in the kinetic mode of the FP instrument by measuring the polarization signal in 1-min intervals over the duration of a 1-h experiment. In a 96-well plate, five separate kinase reactions were run at room temperature in a final volume of 100 μl. All reactions contained 50 μl of the PTK Detection Mix. The first three reactions contained either 1, 5, or 10 ng of c-Src (equal to 0.01, 0.05, and 0.1 ng/μl, respectively), with the following reaction conditions: 50 mM HEPES (pH 7.4), 6 mM $MgCl_2$, 1.25 mM ATP, and 0.125 mg/μl poly(Glu, Tyr) 4:1 substrate. Two control reactions were also run; one did not receive ATP, while the second did not receive the poly(Glu, Tyr) 4:1 substrate. ATP was added last to start each reaction.

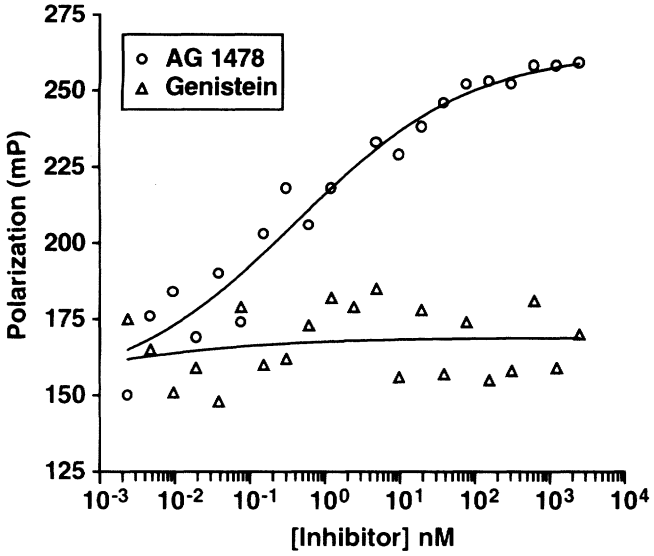

FIG. 8. In a black, round-bottom, 96-well plate, the EGF receptor was preincubated with 2-fold serial dilutions of either AG 1478 or Genistein for 5 min. ATP was then added to start the reactions. Final conditions were 20 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 50 μM Na_3VO_4, 1 nM EGF receptor, 1 mM ATP, 1% DMSO, and 1 mg/ml poly(Glu, Tyr) 4:1 substrate. The reaction was run for 2 h at room temperature and quenched with 20 mM EDTA. An equal volume of the PTK Detection Mix was added and the reaction was incubated for 5 min. The polarization of each well was read on a TECAN Polarion, and nonlinear regression analysis was performed on a semi-log plot of the data to determine the IC_{50} of each compound.

ting program (GraphPad), were determined to be 5.6, 189, 26, and 3.5 nM, respectively (Fig. 4).

In Figure 5, only the 21 compounds with IC_{50} values below 10 μM are shown. Note that higher bars represent higher affinity binding. As hoped, several of these lead compounds exhibited receptor isozyme selectivity. For example, compound 7 has an almost 100-fold higher affinity for ERβ over ERα. Two controls, estradiol (compound 18) and DES (compound 36) are also shown. As expected, both of these compounds are high-affinity competitors of both estrogen receptors.

Kinase assays

As shown in Figure 6, in an endpoint c-Src kinase reaction, polarization decreases with time as products from the kinase reaction (phosphopeptides) displace the tracer from the anti-pY Ab. The polarization changed from an initial value of 250 mP to approximately 100 mP over 10 min. The second set of data, obtained 20 h after the addition of EDTA and the PTK Detection Mix, demonstrates the stability of the quenched kinase reactions and the PTK Detection Mix reagents.

In Figure 7, the kinetic c-Src experiment also shows that polarization decreases with time. These data were collected in real time. The "no ATP" control reaction demonstrates that this reaction, and the subsequent decrease in polarization, is phosphate donor dependent, as expected. The "no substrate" control, when compared to the "no ATP" control, indicates that autophosphorylation of c-Src and/or phosphorylation of the anti-pY Ab do not contribute a detectable amount of competitor phosphotyrosine in this assay.

As Figure 8 shows, the experimentally determined IC_{50} of AG 1478, known to inhibit the EGF receptor at nanomolar concentrations, was 400 pM. Genistein, which is a much weaker inhibitor of the EGF receptor, did not inhibit the EGF receptor at the concentrations tested, as expected.

The results obtained in a kinetic tyrosine phosphatase experiment when using TC PTP are shown in Figure 9. Increasing concentrations of TC PTP result in an increased rate of dephosphorylation, which is indicated by a decrease in polarization. This change in polarization was dependent on the presence of TC PTP and could be completely inhibited by 50 μM Na_3VO_4.

The IC_{50} of an unknown phosphatase inhibitor can also be established with this assay, as illustrated in Figure 10. In this experiment, the IC_{50} of the ubiquitous phosphatase inhibitor Na_3VO_4 was determined to be 4 nM for TC PTP.

Figure 11 shows that each PKC isoform has a different turnover rate for this specific peptide substrate. In the control experiment, which contained no ATP and therefore no PKC reaction products, the polarization value was 225 mP (data not shown). This value represented the maximum possible polarization value for the assay. When various amounts of PKC isoforms were tested in the presence of ATP, the decrease in po-

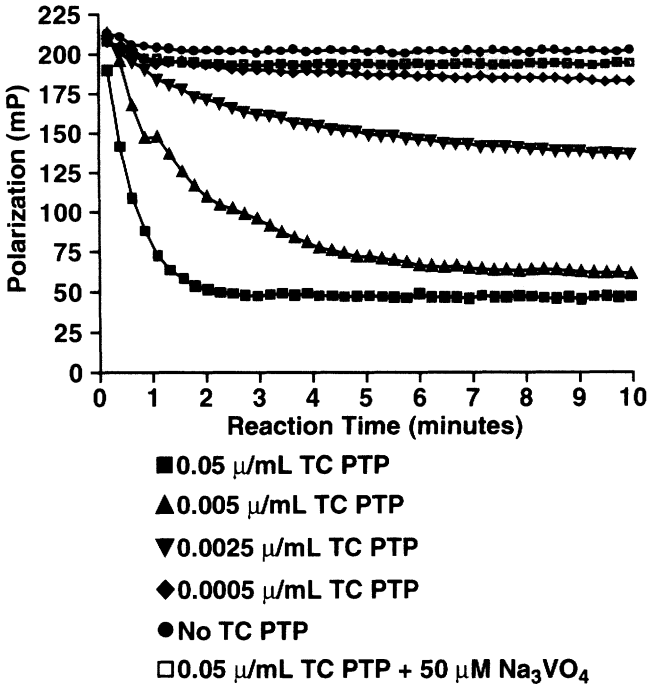

FIG. 9. The enzymatic activity of T-cell protein-tyrosine phosphatase (TC PTP) was measured in a dose-dependent manner by incubating concentration of TC PTP. The TC PTP concentration ranged from 0.05 to 0.0005 U/μl. Each well also contained 50 μl of the PTK Detection Mix in a final volume of 100 μl. Two control assays were performed: one did not receive TC PTP, while the other was supplemented with 50 μM Na_3VO_4, a potent phosphatase inhibitor. In both cases, no dephosphorylation occurred and the polarization remained high throughout the experiment.

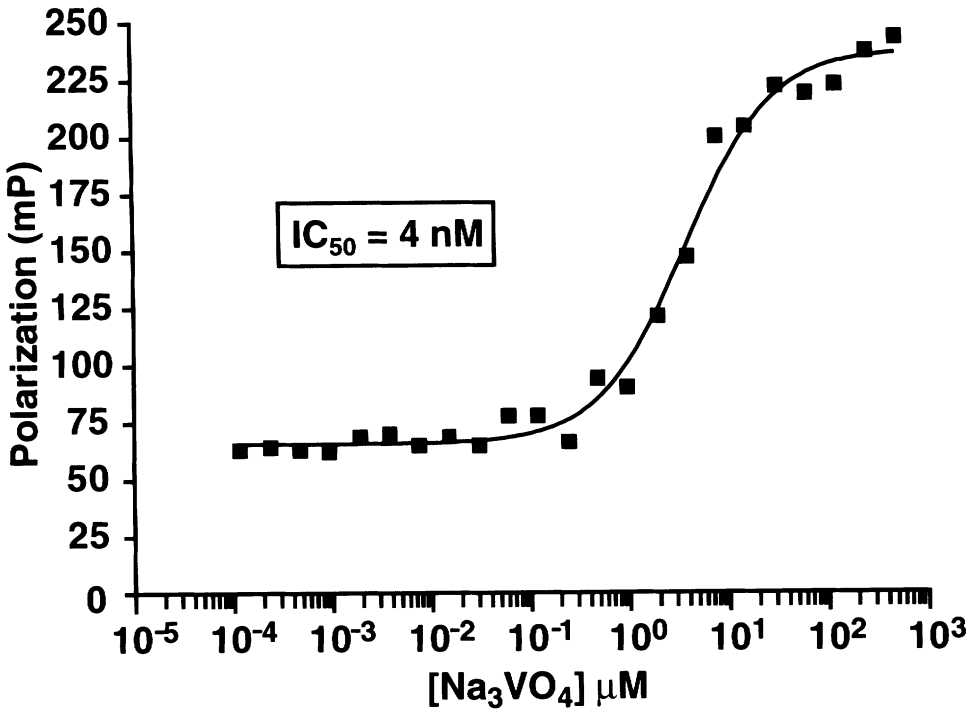

FIG. 10. The IC_{50} value for Na_3VO_4 and TC PTP was determined in the following manner. In a 96-well plate, Na_3VO_4 was serially diluted 2-fold into 24 wells from a starting concentration of 500 nM in a volume of 50 μl. TC PTP was then added to each well so that the final concentration of the enzyme would be 0.005 U/μl per assay. To start the reaction, 50 μl of the PTK Detection Mix was added, and each reaction was incubated for 15 min. At the end of the incubation, 10 μl of 550 μM Na_3VO_4 was added to quench all of the reactions, and the polarization for each sample was measured on a TECAN Polarion. Nonlinear regression analysis of a semi-log plot of the data was used to determine an IC_{50} of approximately 4 nM.

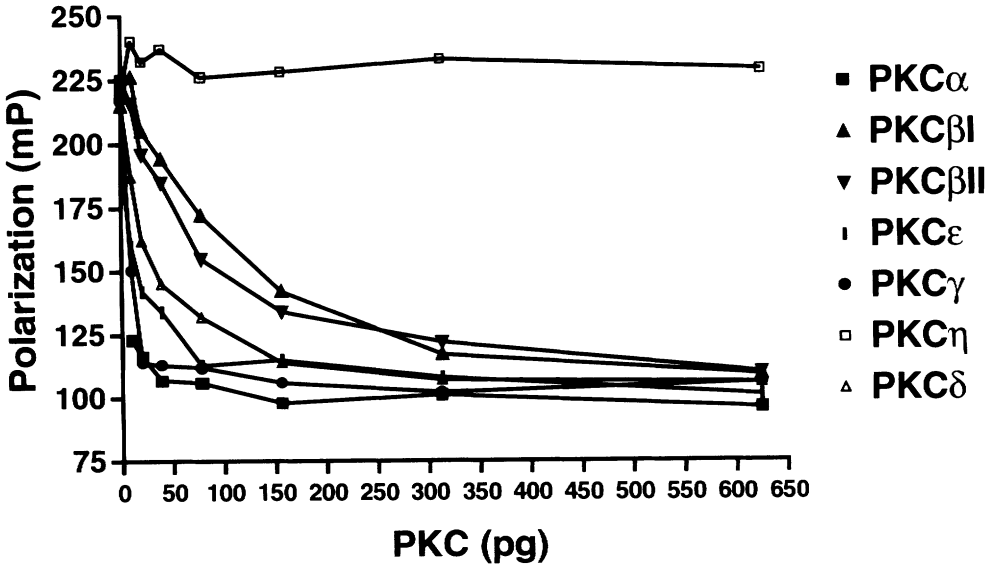

FIG. 11. Seven PKC isoforms were serially diluted in a 96-well plate. The final amount of each PKC ranged from 10 pg to 20 ng per well. After a 5-min incubation with additional PKC reaction components, ATP was added to initiate the reactions. Final reaction conditions (for a 50-μl total reaction volume) were 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 μM $CaCl_2$, 0.1 mg/ml phosphatidylserine, 0.02 mg/ml diacylglycerol, 2 μM peptide substrate. 2× PKC fluorescent phosphopeptide tracer, 50 μM Na_3VO_4, and 10 μM ATP. After a 90-min incubation at room temperature, 50 μl of quench/detection mixture (containing 130 mM EDTA and anti-phosphoserine antibodies) was added to each well to stop the reaction and initiate the competition for antibody binding. Following an additional 30-min incubation at room temperature, the FP of each well was measured on a TECAN Polarion.

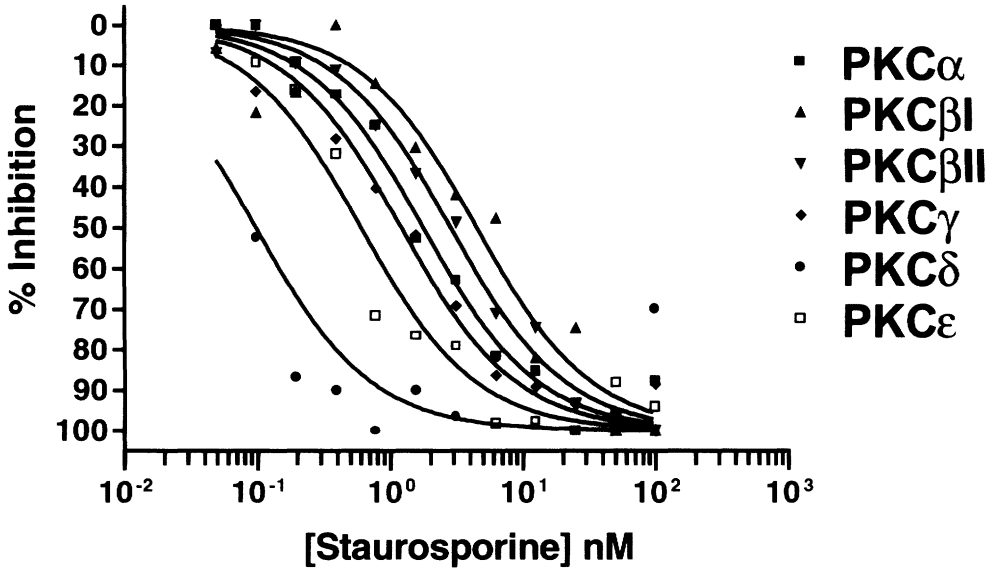

FIG. 12. In a round-bottom, 96-well plate, PKC isoforms (15 ng PKCα, 300 ng PKCβI, 300 ng PKCβII, 17 ng PKCγ, 117 ng PKCδ, and 65 ng PKCϵ) were individually incubated with serial dilutions of staurosporine for 5 min at room temperature. ATP was then added to start the reactions. Final reaction conditions (for 50-μl total reaction volumes) 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 μM $CaCl_2$, 0.1 mg/ml phosphatidylserine, 0.02 mg/ml diacylglycerol, 2 μM peptide substrate, 2× PKC fluorescent phosphopeptide tracer, 50 μM Na_3VO_4, 0.02% NP40, and 10 μM ATP. After a 90-min incubation at room temperature, 50 μl of a quench/detection mixture (containing 130 mM EDTA and the anti-phosphoserine antibody) was added to stop each reaction and initiate the competition for antibody binding. Results were measured on a TECAN Polarion, and nonlinear regression analysis was performed on a semi-log plot of the data. The IC_{50} values of staurosporine were shown to be less than 10 nM for all PKC isoforms tested.

larization was directly proportional to the amount of PKC present in the reaction. Reactions containing the greatest amount of each PKC isoform produced the most unlabeled phosphopeptide products, which ultimately resulted in the lowest polarization values. The results show that 100 pg of most PKC isoforms can complete 50%–70% of the reaction after a 90-min incubation in the presence of ATP. From these data, it was estimated that 15 ng of PKCα, 300 ng of PKCβI, 300 ng of PKCβII, 17 ng of PKCγ, 117 ng of PKCδ, and 65 ng of PKCϵ would give approximately equal amounts of enzymatic activity over a 90-min period under these reaction conditions.

The results in Figure 12 show that the IC_{50} of staurosporine is less than 10 nM for all PKC isoforms tested, with PKCδ being the most potentially inhibited. This correlates well with published data reporting staurosporine IC_{50} values of 9 nM for PKCα, PKCβ, and PKCγ.[35] A staurosporine concentrations increased, the polarization value of the reaction also increased, indicating that PKC activity was being inhibited to a greater extent. The lowest concentrations of staurosporine yielded low polarization values, indicating that the PKC reaction was able to proceed largely (or totally) uninhibited.

DISCUSSION

Estrogen receptor assays

The data show that an FP-based competitive binding assay can be used to screen diverse compounds with a broad range of binding affinities for ER, and that the assay provides a rank ordering of binding affinity values similar to determinations made with conventional assay methods. It should be noted that these binding analyses were carried out under the conditions recommended for HTS application, that is, a relatively high ES2 concentration to overcome potential background fluorescence from test compounds. These conditions are optimal for qualitative identification of ligands in impure samples, but they do not yield accurate affinity measurements for very-high-affinity ligands; for this reason the binding affinities (IC_{50}) for DES and 4-OH-tamoxifen are underestimated using the FP assay. For analytical purposes, use of a lower ES2 concentration (50–100 pM) in a more sensitive single-tube instrument is recommended.

This assay has proven to be fast, robust, and quantitative for the determination of binding affinity for a wide range of estrogen ligands. The quantitative nature of the data and the ability to run assays for multiple receptors in parallel will significantly aid the efforts to develop new ER-responsive drugs with unique activities.

Kinase assays

These data demonstrate that an assay based on FP can be used to detect PTK activity, PKC activity, and phosphatase activity. These assays are simple, rapid, and inexpensive. The screening of unknown kinases, the optimization of substrates, and the testing of reaction conditions are all possible with this assay. The phosphorylated peptide substrates for several tyrosine kinases—including the EGF receptor; insulin receptor; c-Src, Fyn, Lck, and Lyn; and the general kinase substrate poly(Glu, Tyr) 4:1—have been shown to displace the tracer from the anti-pY Ab, while the PKC assay can detect the ac-

tivity of at least seven different PKC isoforms. The principles described here may also be broadly applied to any other modification of a peptide, small protein, or other molecule that can be fluorescently labeled, that can be recognized by an antibody, and that competes for binding with a nonfluorescent molecule.

CONCLUSION

FP is now a method of choice for screening several types of drug targets. The development of new HTS instrumentation and assay methods utilizing the advantages of FP has made FP a technology easily incorporated into HTS. FP should be considered for ligand displacement assays when the ligand can be fluorescently labeled and the receptor is fairly pure and abundant, or when enzyme reaction products can be quantified by immunoassay. The ligand displacement assay approach is being expanded to other nuclear receptors, such as androgen and progesterone receptors. The same competition immunoassay approach used in the PTK and PKC assays is now being used to develop assays for additional serine/threonine kinases.

NOTE ADDED IN PROOF

Since this article was accepted for publication, we have been able to detect the protein kinase activity of cAMP-dependent protein kinase (PKA), calmodulin-dependent protein kinase (CamKII), and glycogen synthase kinase-3β (GSK-3β) with the reagents, methods, and sensitivity similar to that described herein for the PKC assays.

REFERENCES

1. Drews, J. (1996). Genomic sciences and the medicine of tomorrow. *Nat. Biotechnol.* 14:1516–1517.
2. Gallop, M.A., Barrett, R.W., Dower, W.J., Fodor, S.P., Gordon, E.M. (1994). Applications of combinatorial technologies to drug discovery. 1. Background and peptide combinatorial libraries. *J. Med. Chem.* 37:1233–1251.
3. Pope, A.J., Haupts, U.M., Moore, K.J. (1999). Homogeneous fluorescence readouts for miniaturized high-throughput screening: Theory and practice. *Drug Discov. Today* 4:350–362.
4. Heyduk, T., Ma, Y., Tand, H., Ebright, R.H. (1996). Fluorescence anisotropy: Rapid, quantitative assay for protein-DNA and protein-protein interaction. *Methods Enzymol.* 274:492–503.
5. Jameson, D.M., Sawyer, W.H. (1995). Fluorescence anisotropy applied to biomolecular interactions. *Methods Enzymol.* 246: 283–300.
6. Checovich, W.J., Bolger, R.E., Burke, T. (1995). Fluorescence polarization—a new tool for cell and molecular biology. *Nature* 375:254–256.
7. Bolger, R., Wiese, T.E., Ervin, K., Nestich, S., Checovich, W. (1998). Rapid screening of environmental chemicals for estrogen receptor binding capacity. *Environ. Health Perspect.* 106: 551–557.
8. Lundblad, J.R., Laurance, M., Goodman, R.H. (1996). Fluorescence polarization analysis of protein-DNA and protein-protein interactions. *Mol. Endocrinol.* 10:607–612.
9. Ozers, M.S., Hill, J.J., Ervin, K., Wood, J.R., Nardulli, A.M., Royer, C.A. Gorski, J. (1997). Equilibrium binding of estrogen receptor with DNA using fluorescence anisotropy. *J. Biol. Chem.* 272:30405–30411.
10. Wu, P., Brasseur, M., Schindler, W. (1997). A high-throughput STAT binding assay using fluorescence polarization. *Anal. Biochem.* 249:29–36.
11. Bolger, R., Checovich, W. (1994). A new protease activity assay using fluorescence polarization. *Biotechniques* 17:585–589.
12. Bolger, R., Thompson, D. (1994). A quantitative RNase assay using fluorescence polarization. *Am. Biotechnol. Lab.* 12:113–116.
13. Turner, R.T., Riggs, B.L., Spelsberg, T.C. (1994). Skeletal effects of estrogen. *Endocr. Rev.* 15:275–300.
14. Ciocca, D.R., Roig, L.M. (1995). Estrogen receptors in human nontarget tissues: Biological and clinical implications. *Endocr. Rev.* 16:35–61.
15. Farhat, M.Y., Lavigne, M.C., Ramwell, P.W. (1996). The vascular protective effects of estrogen. *FASEB J.* 10:615–624.
16. Kuiper, G.G.J.M., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustadsson, J.-A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. U.S.A.* 93:5925–5930.
17. Nilsson, S., Kuiper, G., Gustafsson, J.-A. (1998). ERβ: A novel estrogen receptor offers the potential for new drug development. *Trends Endocrinol. Metab.* 9:387–395.
18. Korach, K.S., McLachlan, J.A. (1995). Techniques for detection of estrogenicity. *Environ. Health Perspect.* 103:5–8.
19. Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Serrano, F.O. (1995). The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environ. Health Perspect.* 7:113–122.
20. Reese, J.C., Katzenellenbogen, B.S. (1991). Mutagenesis of cysteines in the hormone binding domain of the human estrogen receptor: Alterations in binding and transcriptional activation by covalently and reversibly attaching ligands. *J. Biol. Chem.* 266:10880–10887.
21. Haggblad, J., Carlsson, B., Kivela, P., Siitari, H. (1995). Scintillating microtitration plates as a platform for determination of [^{3}H]estradiol binding constants for hER-HBD. *Biotechniques* 18:146–151.
22. Hunter, T. (1995). Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* 80:225–236.
23. Hunter, T. (1998). The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: Its role in cell growth and disease. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 353:583–605.
24. Pawson, T. (1995). Protein modules and signalling networks. *Nature* 373:573–580.
25. von Deimling, A., Louis, D.N., Wiestler, O.D. (1995). Molecular pathways in the formation of gliomas. *Glia* 15:328–338.
26. Friess, H., Berberat, P., Schilling, M., Kunz, J., Korc, M., Buchler, M.W. (1996). Pancreatic cancer: The potential clinical relevance of alterations in growth factors and their receptors. *J. Mol. Med.* 74:35–42.
27. Wong, A.J., Ruppert, J.M., Bigner, S.H., Grzeschik, C.H., Humphrey, P.A., Bigner, D.S., Vogelstein, B. (1992). Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc. Natl. Acad. Sci. U.S.A.* 89:2965–2969.
28. Eppstein, D.A., Marsh, Y.V., Schryver, B.B., Bertics, P.J. (1989). Inhibition of epidermal growth factor/transforming growth factor-alpha-stimulated cell growth by a synthetic peptide. *J. Cell. Physiol.* 141:420–430.
29. Baum, E.Z., Johnston, S.H., Bebernitz, G.A., Gluzman, Y. (1996).

Development of a scintillation proximity assay for human cytomegalovirus protease using ^{33}phosphorus. *Anal. Biochem.* 237:129–134.

30. Kolb, A.J., Kaplita, P.V., Hayes, D.J., Park, Y.-W., Pernell, C., Major, J.S., Mathis, G. (1998). Tyrosine kinase assays adapted to homogeneous time-resolved fluorescence. *Drug Discov. Today* 3:333–342.
31. Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science* 233:305–312.
32. Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258:607–614.
33. Dekker, L.V., Parker, P.J. (1994). Protein kinase C—a question of specificity. *Trends Biochem. Sci.* 19:73–77.
34. Hu, H. (1996). Recent discovery and development of selective protein kinase C inhibitors. *Drug Discov. Today* 1:438–447.
35. Tamaoki, T., Nomoto, H., Takahaski, I., Kato, Y., Mori:noto, M. Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* 135:397–402.

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