Transscreener™: screening enzymes involved in covalent regulation

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Enzymes that catalyse group transfer reactions comprise a significant fraction of the human proteome and are a rich source of drug targets because of their role in covalent regulatory cycles. Phosphorylation, glycosylation, sulfonation, methylation and acetylation represent some of the key types of group transfer reactions that modulate the function of diverse biomolecules through covalent modification. Development of high-throughput screening methods for these enzymes has been problematic because of the diversity of acceptor substrates. Recently, the authors developed a novel assay platform called Transcreener™ that relies upon fluorescence detection of the invariant reaction product of a group transfer reaction, usually a nucleotide. This platform enables screening of any isoform in a family of group transfer enzymes, with any acceptor substrate, using the same assay reagents.

1. Overview of the market

1.1 Importance of covalent regulation

Covalent modification of biomolecules plays a central role in signal transduction at all levels in an organism [1,2]. Post-translational modifications such as phosphorylation and glycosylation embellish most eukaryotic proteins, controlling their localisation, enzymatic activity, and interaction with other proteins in signalling networks [3,4]. Chromatin and DNA methylation and acetylation are the primary mechanisms for epigenetic regulation of gene expression [5,6]. Small-molecule modifications, such as glucuronidation and sulfation control the stability of circulating hormones and xenobiotics and their activity within target tissues [7,8] (**Table 1**).

Most covalent modifications are the result of enzymatically catalysed group transfer reactions of the general form: Donor-X + Acceptor → Donor Product + Acceptor-X. In many cases, the adduct is activated by a high energy bond to a nucleotide or a nucleotide-containing cofactor; for example, adenosine triphosphate (ATP) for phosphorylation. The enzymes that catalyse these reactions are generally classified based on their selectivity for acceptor substrates, which varies extensively within a family. For instance, protein kinases are divided into two broad classes: serine/threonine kinases and tyrosine kinases, and the exquisite selectivity of individual kinases is defined by the sequence of the acceptor protein in the region surrounding the target amino acid. Enzymes that modify small molecules, especially those involved in xenobiotic metabolism, tend to be more promiscuous (i.e., they recognise a broader range of chemical structures) than those that modify proteins but still generally target one or a few classes of structurally related acceptors. Most types of group transfer reactions occur both on macromolecules and small molecules, thus acceptor specificity within a family can be extremely broad (**Table 1**). The absolute number of group

transfer enzymes in humans is not known, but from the available genetic data, they constitute at least 10% of coding sequences in the genome [9].

The primary role of enzymes that catalyse group transfer reactions is to regulate the stability and function of other biomolecules; thus, they are natural targets for therapeutic intervention. Moreover, in many cases covalent modifications are reversible because of the presence of hydrolase enzymes, and the resulting enzymatic cycle provides a dynamic, tunable control mechanism that can operate either as an on-off switch or a rheostat [10]. Such regulatory cycles are important focal points for the control of cell signalling. For these reasons, enzymes that catalyse group transfer reactions are increasingly being incorporated into pharmaceutical high-throughput screening (HTS) programmes.

Next to G-protein-coupled receptors, protein kinases are the most intensively screened target class [11], and glycosyltransferases, methyltransferases and sulfotransferases are emerging target classes [6,12,13]. However, the broad acceptor specificity within a family complicates their incorporation into HTS because most assay methods rely on detection of the covalently modified product; (e.g., phosphopeptides for protein kinases [see Section 2]). This requires the development and use of different detection reagents for individual enzymes, which slows screening of new family members and hampers inhibitor selectivity profiling across diverse enzymes within a family. The Transcreener™ HTS assay platform was developed to overcome this technical hurdle. Because it relies on detection of the invariant product for a group transfer reaction (**Figure 1**), it enables screening of all members of a family of group transfer enzymes using the same detection reagents. Below, the authors review the importance of the group transfer enzyme families for which Transcreener™ reagents have been developed.

1.2 Protein kinases

Protein kinases catalyse the transfer of the terminal phosphate group from ATP or GTP to serine, threonine or tyrosine residues of acceptor proteins. They comprise one of the largest protein families in the human genome with > 400 members [14-16]. In the broadest senses, they can be divided into serine/threonine or tyrosine kinases and soluble enzymes or transmembrane receptors. In the most recent and comprehensive genomic analyses, 428 human kinases were identified that comprise 8 different homology groups, which also reflect differences in substrate specificity, structure/localisation and/or mode of regulation [15]. For instance, there are 84 members of the tyrosine kinase group, which includes both transmembrane growth factor receptors such as epidermal growth factor receptor and soluble enzymes such as the Src kinases, 61 members of the cyclic nucleotide-dependent group, Ser/Thr kinases which include the protein kinase C isoforms, and 45 members of the 'STE' group, which includes the components of the mitogenic MAPK signalling pathway [15].

Kinases are ubiquitous regulators of intracellular signal transduction pathways and, as such, have come under intense focus by pharmaceutical companies searching for more selective therapies for a broad range of diseases and disorders [3,11,17], especially cancers. Site-specific protein phosphatases can catalyse the removal of phosphates from proteins, so that a tunable covalent modification cycle modulates protein function [3,17]. Intracellular targets for phosphorylation include other kinases, transcription factors, structural proteins such as actin and tubulin, enzymes involved in DNA replication and transcription, protein translation and metabolic enzymes [3]. Phosphorylation can cause changes in protein catalytic activity, specificity, stability, localisation and association with other biomolecules. Simultaneous phosphorylation at multiple sites on a protein, with different functional consequences, is common and central to the integration of signalling pathways [3].

1.2.1 The kinase HTS bottleneck

Recent clinical success with small-molecule kinase inhibitor drugs, most notably imantinib mesylate, a Bcr-Abl inhibitor used to treat chronic myelogenous leukaemia, has generated intense interest in the kinase family [18]. However, the ability to validate and pursue new kinases for drug discovery is being hampered by a lack of screening assays capable of accommodating diverse kinase isoforms. Most kinase assay methods rely on detection of a tagged phosphopeptide (see Section 2), and significant development or optimisation is required to accommodate new peptide sequences. Protein kinases recognise specific linear sequences of their target proteins that often occur at beta bends. In general, amino acids that flank the phosphorylated residue for three to five residues on either side define a phosphorylation site. The Phospho.ELM database [101], which compiles known kinase phosphorylation sites, contains entries for 195 eukaryotic kinases (mostly human), less than half of the total kinases. Moreover, most, if not all, of these specificity profiles are incomplete. Although there is significant overlap in substrate specificity among related kinases, there is no consensus sequence that is phosphorylated by a large number of kinases [19]. The time and money required to develop phosphopeptide-based assay methods for kinases with novel recognition sequences is slowing the identification of selective inhibitors for validating new kinase targets or as potential lead molecules.

The assay development bottleneck extends further downstream in the discovery process as well. There is a great deal of structural similarity between kinase active sites, and a significant effort goes toward identifying the most selective inhibitors to minimise side effects from off-target activity. At some point following a primary screen, secondary screens are often run against a panel of 10 – 100 kinases to obtain selectivity profiles for the most promising hits. These profiling studies generally include potency determinations for compounds where significant off-target activity is observed. The available nonradioactive HTS assay methods are not suitable for quantitative assays with a broad spectrum or kinases, and studies
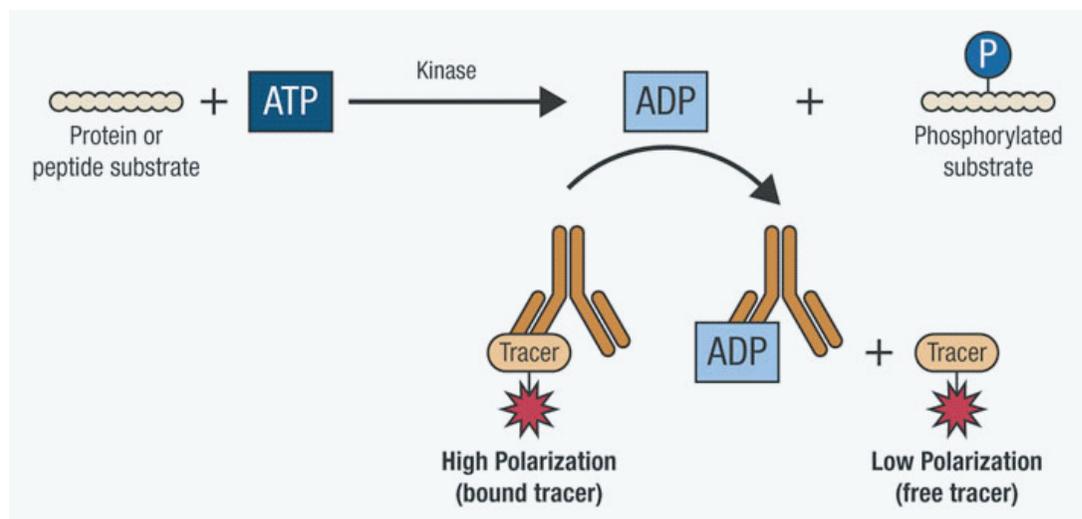
Figure 1. The Transcreener™ kinase assay is based on detection of the donor product – ADP for kinases – by fluorescence polarisation immunoassay. ADP produced in the kinase reaction displaces a fluorescent tracer from antibody resulting in a decrease in its fluorescence polarisation.

Table 1. Examples of group transfer enzyme families.

Family	Number of genes	Donor	Donor product	Acceptors
Protein kinases	> 400	ATP	ADP	Proteins
Glycosyltransferases	> 200	NDP-sugar	NDP (UDP is most common)	Proteins, lipids, small molecules
Methyltransferases	> 50	S-adenosylmethionine	S-adenosylhomocysteine	Proteins, small molecules, DNA, RNA
Sulfotransferases	> 50	Phosphoadenosine-phosphosulfate	Phosphoadenosine-phosphosulfate	Proteins, small molecules
Acetyltransferases	> 10	Acetyl-coenzyme A	Coenzyme A	Proteins, small molecules,
ADP-ribosyltransferases	ND	NAD	Nicotinamide	Proteins, DNA

ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; ND: Not determined; NAD: Nicotinamide adenine dinucleotide; NDP: Nucleotide diphosphate; UDP: Uridine diphosphate.

have shown that there are significant differences in the identity and potency of hits determined with different HTS assay methods [20]. For these reasons, the bulk of selectivity profiling is done using the traditional ^{33}P-radioassays. Most pharmas contract this work at significant expense and inconvenience to service providers who are willing to assume the regulatory and disposal requirements.

1.3 Glycosyltransferases

There are > 200 glycosyltransferases encoded in the human genome, and most use uridine diphosphate (UDP)-activated sugars as the donor [11]. From a functional standpoint, the reactions they catalyse can be divided into three major types: biosynthesis of disaccharides or polymers such as starch or hyaluronan, post-translational modification of proteins, and

glucuronidation of small molecules, including endogenous hormones and xenobiotics [11].

All three types of glycosyltransferases are of interest in drug discovery. The role of the ~ 15 hepatic UDP-glucuronosyltransferases (UGTs) in drug metabolism has been an area of focus for several years [8,21]. More recently, protein glycosyltransferases and those involved in synthesis of biopolymers have come under focus as therapeutic targets, for cancer [22,23] (**Table 2**) and lysosomal storage diseases [24]. Bacterial glycosyltransferases involved in cell wall synthesis are also being targeted for development of new anti-infectives [25,26].

1.3.1 Glucuronidation of drugs

Aside from P450-depedent oxidation, glucuronidation is the most important route of hepatic drug metabolism [27].

Table 2. Examples of glycosyltransferases involved in tumourigenesis.

Glycosyltransferase	Roles in cancer
Hyaluronan synthases (3 isoforms)	Hyaluranon is major ECM component; affects cell proliferation, invasion, angiogenesis [29]
N-acetylglucosamine protein O-glycosyltransferase	Mitogenic effects via glycosyation of oncogenes and tumour supressors [71]
N-acetylglucosamine protein N-glycosyltransferase (GnTV)	Elevated N-linked glycans on cell surface proteins promotes cell detachment and invasion [72]
Sialyltransferases (ST6Gal-I)	Elevated polysialic acid and sialylation of cell surface proteins promotes cell detachment and invasion [73]

ECM: Extracellular matrix.

Glucuronidation usually facilitates excretion of drugs by increasing their aqueous solubility, but in some cases it increases drug activity. A broad spectrum of drugs are eliminated or activated by this route, including nonsteroidal anti-inflammatories, opioids, antihistamines, antipsychotics and antidepressants [8,21]. Pharmaceutical companies have an immediate need for better methods to determine whether their potential drug candidates will be glucuronidated, and if so, by which UGT isoform. The current state of the art is isolation and detection of glucuronides from *in vitro* enzymatic reactions using liquid chromatography/ mass spectometry, a method not suited for profiing large numbers of compounds. Fluorescent probe substrates and assay methods for detecting glucuronidation have been developed, but have not been widely adapted because they cannot be used with all UGT isoforms [28,102].

1.3.2 Glycans in cancer

Glycans inside and outside the cell play significant roles at all pathophysiological stages of tumour development [22,23]. For example, hyaluronan, a polymer of repeating disaccharide units of D-glucuronic acid and N-aetylglucosamine, is an important component of the extracellular matrix (ECM) that has been linked with many tumour-related activities including cell adhesion and motility, cell growth and differentiation, and angiogenesis [29]. Elevated levels of hyaluronan and hyaluronan synthase enzymes have been linked with high metastatic potential in prostate and breast cancer [29], with tumour vascularisation in prostate cancer [30] and with poor prognosis in multiple myeloma and ovarian cancer [31]. N-acetylglucosaminyltransferase V is a protein glycosyltransferase that is overexpressed in many malignant cells [23]. The resulting higher levels of β1,6-branched N-linked glycans on the cell surface proteins decreases cell–cell and cell–ECM interactions resulting in increased tumour cell motility and invasion across basement membranes [32].

Currently, efforts to identify glycosyltransferase inhibitors are largely dependent on assays that are specific for the glycosylated products, though more flexible methods have recently begun to emerge [33,34]. Because of the tremendous diversity in acceptor substrates and glycosylation sites, validation of glycosyltransferases as drug targets and identification and optimisation of lead molecules will likely experience bottlenecks similar to those encountered with protein kinases.

1.4 Sulfotransferases

Sulfotransferase enzymes (SULTS) catalyse the conjugation of sulfonate groups onto a variety of xenobiotic and endogenous substrates, primarily at hydroxyl groups, to form a sulfate, but also at some aromatic amines resulting in sulfamate formation [35]. 3′-Phosphoadenosine 5′-phosphosulfate (PAPS) serves as the donor molecule for the activated sulfate. The product after sulfate transfer is the 3′,5′-bisphosphate adenine ribonucleotide, known as phosphoadenosine phosphate (PAP).

There are two major classes of SULTs in humans that differ in solubility, size, subcellular distribution, and share < 20% sequence homology [12]. The 37 membrane-bound enzymes are located in the golgi apparatus and sulfonate large endogenous molecules, such as heparan, glycosaminoglycans and protein tyrosines [7]. The 12 cytosolic sulfotransferases, conjugate small-molecular weight xenobiotics and hormones. The golgi-associated sulfotransferases are being targeted for cancer and inflammation because they play roles similar to glycosyltransferases in cell–cell and cell–matrix interactions [12]. In this review, the authors focus on the importance of the cytosolic sulfotransferases in drug metabolism and regulation of hormone activity.

1.4.1 Xenobiotic sulfation

Sulfation is the second most prevalent form of conjugative metabolism after glucuronidation [36-38]; together these two reactions account for more than two-thirds of total conjugative xenobiotic metabolism [39]. Moreover, there is significant genetic variability in SULT genes, with functional consequences. Nonsynonymous polymorphisms (i.e., mutations that result in a change in amino acid sequence) have been identified in more than half of the xenobiotic metabolising SULTs [40-44], and many of these result in differences in enzyme activity levels, stability, or other properties [40-44] with potential clinical relevance. For example, homozygous expression of the SULT1A1*2 allele has recently been linked with a threefold increased risk of death in breast cancer patients receiving tamoxifen [45]. The high frequency of functionally significant SULT polymorphisms and their association with differences in drug response highlights the need for implementing a pharmacogenomic approach for drug metabolism by sulfation. Recombinant SULT isoforms have been

Table 3. Substrate specificity of human cytosolic sulfotransferase isoforms (SULTs), cellular receptors that are affected by the resulting sulfoconjugates, and their disease links.

SULT	Substrates	Receptors affected	Disease/disorder
1A1	Simple phenols, catechols, minoxidil, paracetamol, hydroxylarylamines, iodothyronines, estradiol, anti-estrogens	TR, ER	Sulfates many phenolic drugs
1A2	Simple phenols, catechols	NA	NA
1A3	Catecholamines: dopamine, adrenaline, noradrenaline, catechols, tyramine, 5-hydroxytryptamine, salbutamol	α- and β-adrenergic receptors	Cardiogenic shock, arrhythmias, infarction
1C1,2	N-hydroxy-2-acetylaminofluorene	NA	NA
1B1	Simple phenols, catechols, iodothryonines	TR	Hypo- and hyperthyroidism, depression
1E1	Estrogens, anti-estrogens, androgens, pregnenolone, minoxidil, iodothyronines, α-napthol	ER, GABA$_A$-R, TR, NMDA-R, Sigma 1-R, MAP-2	Breast and prostate cancer, osteoporosis
2A1	DHEA, pregnenolone, cortisol, testosterone, estradiol, bile salts	AR, GR, MR, ER, GABA$_A$-R, NMDA-R, Sigma 1-R, MAP-2	Prostate cancer, cognition, memory, psychosis
2B1a,b	DHEA, pregnenolone, cholesterol, epiandrosterone, androstenediol, genestein	ER, GABA$_A$-R, NMDA-R, MAP-2, PKC, LXR	Skin cancer, depression atherosclerosis
4A1	Iodothyronines, estrone, nitrophenol, napthol	ND	Specifically expressed in brain
6B1	Unknown	ND	ND

AR: Androgen receptor; ER: Estrogen receptor; GABAA-R: γ-Aminobutyric acid receptor type A; GR: Glucocorticoid receptor; LXR: Liver X receptor; MAP: Microtubule-associated protein; MR: Mineralcorticoid receptor; NA: Not available; ND: Not determined; NMDA-R, N-methyl-D-aspartate receptor; PKC: Protein kinase C; Sigma-1-R, Sigma receptor, type 1; TR: Thyroid receptor.

expressed and purified, and could be used to screen diverse molecules for sulfation if suitable assay methods were available.

1.4.2 Regulation of hormones by sulfation

Intertwined with its role in drug metabolism, sulfation also reversibly regulates the activity of many endogenous signalling molecules, including steroid hormones and catecholamines, that are involved in the pathology of diseases such as breast cancer [46,47], cardiovascular disease [48,49], and depression [50] (**Table 3**). In contrast to its general effect of increasing the clearance rate for drugs and other xenobiotics, sulfation normally increases the stability of circulating hormones [7]. And perhaps more importantly, sulfation causes changes in the activity of biomolecules, often by altering their affinity for cellular receptors [7,51,52]. Because the sulfate can be removed by cellular sulfatases, sulfation can serve as an on-off switch for receptor ligands much as phosphorylation plays that role for proteins. In this regard, sulfation is emerging as an important way of regulating the activity of hormones and other signalling molecules at their final site of action, within target cells [7,52]. For instance,

estrogen sulfoconjugates are the main circulating forms of the hormone, and they require desulfation in the cell in order to bind and activate the estrogen receptor [52]. The involvement of sulfation in disease pathology and drug metabolism raises the potential for unintended interactions between drugs and hormones competing for the same sulfotransferase and underscores the importance of delineating the selectivity of the SULTS during drug discovery.

1.5 Market need

The success of kinase inhibitor drugs such as imantinib mesylate is increasing the demand for more rapid movement of diverse kinase enzymes into HTS, both as new targets and for inhibitor selectivity profiling. At the same time, because of their emerging roles in disease and their importance to drug metabolism, there is a strong unmet need for HTS assay methods for glycosyltransferases, sulfotransferases and other group transfer enzyme families. The Transcreener™ HTS platform was developed to overcome these technical hurdles and thereby accelerate the discovery and development of more selective drugs.
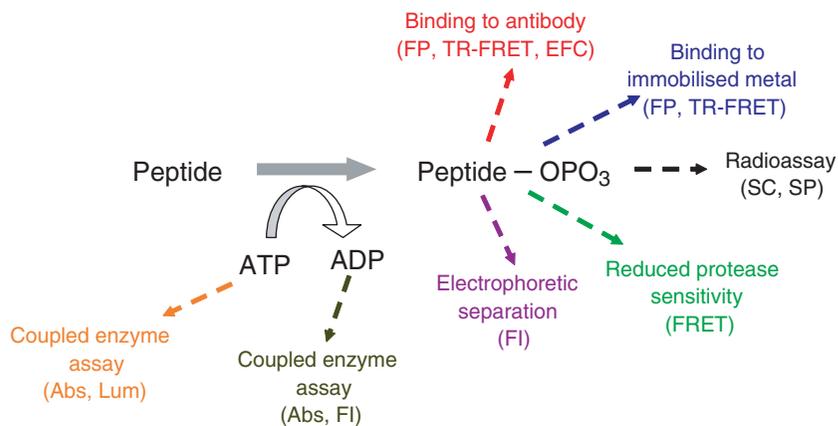
Figure 2. Alternative technologies: Protein kinase assay methods.

Abs: Absorbance; EFC: Enzyme fragment complementation; FI: Fluorescence intensity; FP: Fluorescence polarisation; Lum: Luminescence; SC: Scintillation counting; SP: Scintillation proximity; TR-FRET: Time-resolved fluorescence resonance energy transfer.

2. Alternative technologies: protein kinase assay methods

Prior to development of the Transcreener™ assays described here, protein kinases were the only family of group transfer enzymes for which robust HTS enzymatic assays were available. Traditionally, kinases have been assayed by filter capture or precipitation of radiolabelled peptide or protein substrates produced using ^{32}P-ATP or ^{33}P-ATP as donor. This method is still used for selectivity profiling by service providers because it is generic, but most pharmas avoid radioassays in HTS because of the associated regulatory and disposal issues. Binding of tagged phosphopeptides to antibody or immobilised metal ions are the most common assay methods used for kinases in HTS [53,54]; binding is coupled to a variety of fluorescent and chemiluminescent detection modes [55-57]. Phosphopeptide detection methods are not generic; (i.e., different reagents and/or conditions are required for different kinases). In addition, the use of native protein acceptor substrates is restricted to varying degrees by the different phosphopeptide detection methods. Microfluidics-based kinase assays that rely on electrophoretic separation of phosphorylated peptides have been developed [58], but in practice, the kinase assays are often run in plates, and products are then transferred to microfluidic devices for separation – a cumbersome process for an HTS format. ATP and ADP detection are truly generic kinase assay methods. Luciferase has been used to monitor ATP consumption [59], but this approach requires a high level of substrate consumption to generate adequate signal to noise. Coupled enzyme assays that generate a chromophore have been employed for many years to detect ADP produced in kinase reactions [60], but this method has not been widely adapted in HTS because the sensitivity and signal–noise ratio of absorbance-based detection is low. **Figure 2** summarises these alternative methods.

3. How the technology works

Transcreener™ is a universal HTS assay platform that relies on immunodetection of the invariant reaction product – the donor product – of a group transfer reaction (**Figure 1**). This enables screening of all of the enzymes within a group transfer family using the same assay and reagents. Notably, it also allows direct screening of group transfer family members with potential acceptor substrates, which is especially relevant for analysing drug and xenobiotic conjugation in preclinical ADME studies. It is a homogenous or 'mix-and-read' assay platform that can be coupled with several detection modes, including the most common fluorescence modes currently used for HTS, such as fluorescence polarisation, and time resolved fluorescence resonance energy transfer.

The first generation Transcreener™ assays rely on fluorescence polarisation immunoassay (FPIA) detection of the donor product – ADP in the case of kinases – that is produced in stoichiometric amounts with phosphorylated polypeptide (**Figure 1**). FP is used to study molecular interactions by monitoring changes in the apparent size of fluorescently-labelled or inherently fluorescent molecules [61,62]. When a small fluorescent molecule (tracer) is excited with plane-polarised light, the emitted light is largely depolarised because the molecule rotates rapidly in solution during the fluorescence event (the time between excitation and emission). Binding of the tracer to an antibody increases its effective molecular volume and slows its rotation sufficiently to emit more light in the same plane in which it was excited. The Transcreener™ platform employs a competitive fluorescence polarisation immunoassay, in which the donor product (e.g., ADP) produced from the group transfer reaction competes with the tracer (fluorescently tagged donor product) for binding to an antibody. In this format, the starting polarisation is high, and decreases as the reaction proceeds. FP is
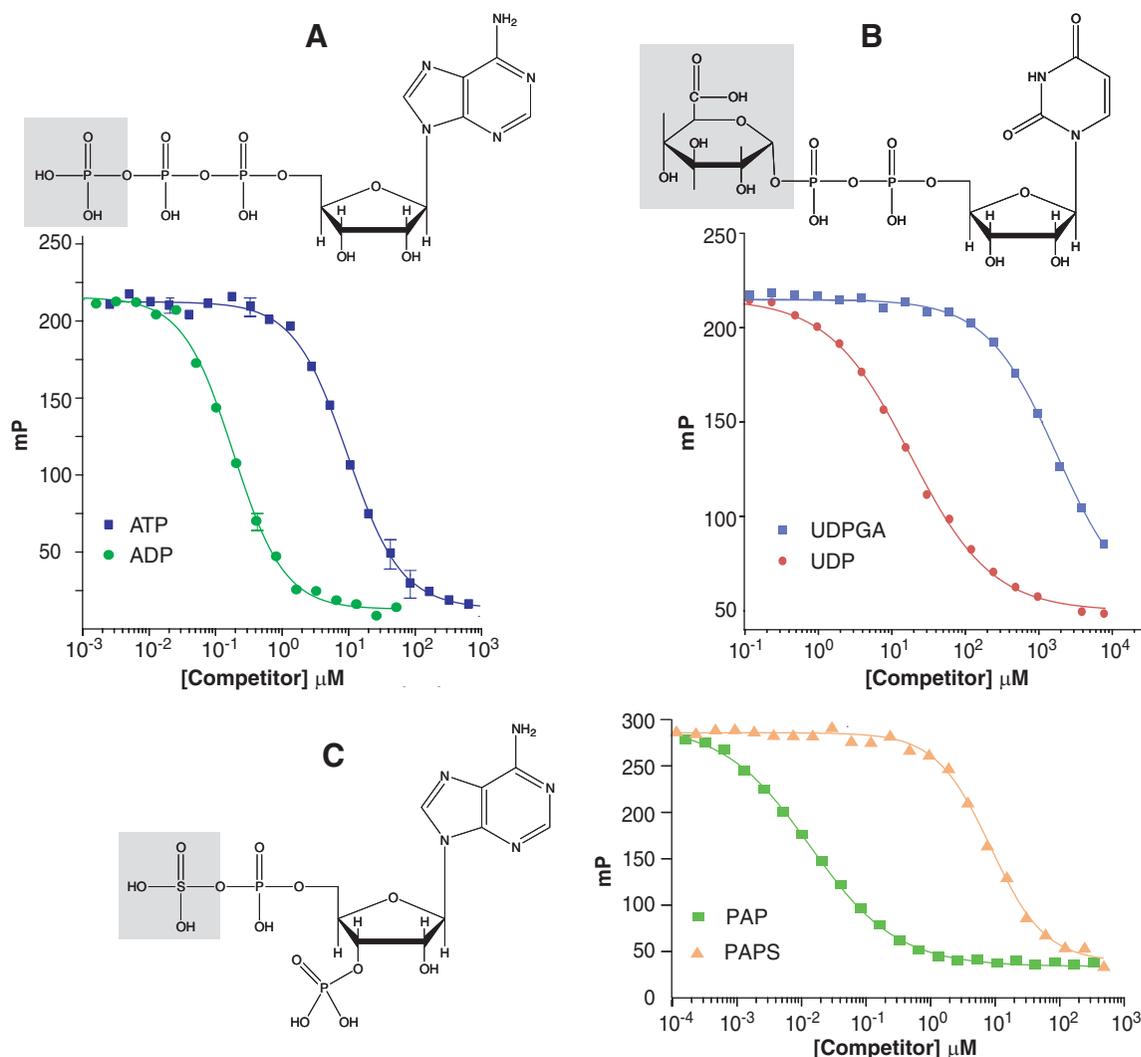
Figure 3. Fluorescence polarisation competition curves for donor and donor product nucleotides with antibodies raised against (A) ADP, (B) UTP and (C) PAP. Structures of the corresponding donor molecules are shown by each graph with the group that is transferred in the reaction greyed-out. Near saturating amounts of each antibody were added to 2 nM tracer (fluorescent conjugates of ADP, UTP or PAP) resulting in high polarisation values. As increasing amounts of unlabelled nucleotides are added, the tracer is displaced from antibody causing its polarisation to decrease. The IC_{50} values observed for each of the donor product/donor pairs in these experiments were, respectively: 0.187 μM and 9.7 μM for ADP and ATP, 17.8 μM and 1.77 mM for UDP and UDPGA, 13.5 nM and 8.56 μM for PAP and PAPS.

PAP: Phosphoadenosine-phosphosulfate; PAPS: Phosphoadenosinephosphate.

broadly accepted in HTS, with FPIA for phosphopeptide detection already in extensive use for kinases [54,63,64] and FP-based assays also in use for a number of other enzymatic and ligand binding reactions [62,64].

Thus far, proof-of-concept has been shown for Transcreener™ assays for protein kinases, glycosyltransferases, sulfotransferases. Assays for other families of group transfer enzymes, including methyltransferases and acetyltransferases are under development. The key reagents are an antibody specific for the donor product and a fluorescent conjugate to the donor product – called a tracer – that retains its antibody binding properties. The major technical hurdle is the

development of antibodies that bind the donor product (ADP, UDP etc.) with high selectivity over the donor molecule. In addition to the structural similarity of the donor product to the donor, the challenges include the small size and lability of the antigens. As shown in **Figure 3**, antibodies for three donor molecules, ADP, UDP and PAP, have been generated with nanomolar affinities for tracer and 50 to 800-fold selectivities for donor product over donor.

Several different conjugates of nucleotides to carrier proteins and fluors were synthesised using similar chemistry and the resulting antibodies and tracers were codeveloped in an iterative fashion. The chemistry of hapten conjugation to
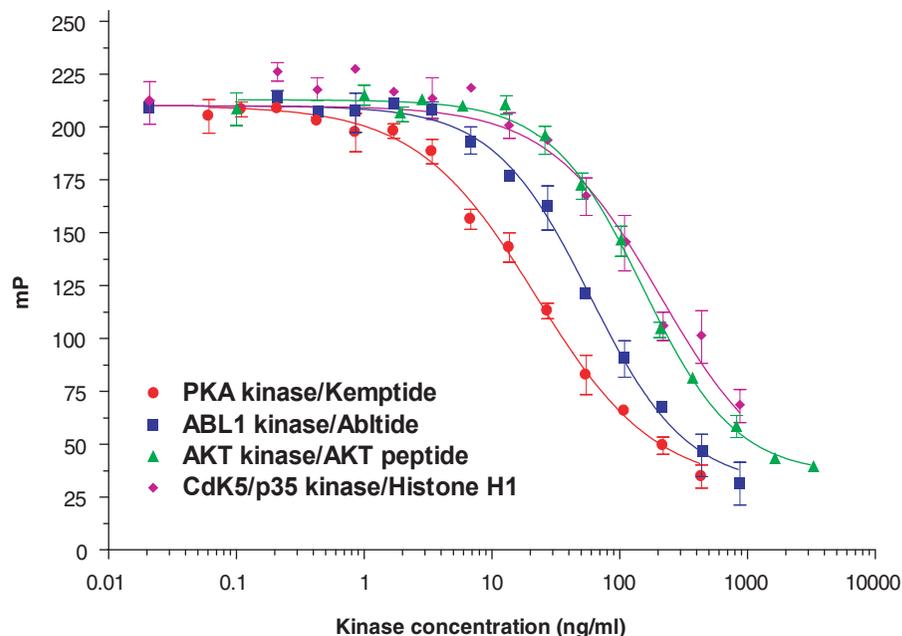
Figure 4. A single set of reagents can be used to assay any kinase using peptide or protein substrates. Enzyme titration curves for three Ser/Thr kinases and one Tyr kinase (ABL1) were prepared by adding an equal volume of the Transcreener™ ADP-detection mix after a 60 minute 25°C kinase reaction. Polarisation decreases with increasing kinase as the ADP produced displaces the tracer from antibody. Kinases were serially diluted twofold in buffer containing the following substrates: PKA = kemptide; Abl = Abltide, CDK5/p35 = Histone H1, and AKT/PKBα = AKT/SGK substrate peptide.
CDK: Cyclin-dependent kinase; PKA: Protein kinase A.

carrier protein plays a critical role in the selectivity and affinity of the antibody. In all cases, nucleotides were conjugated via the base to maximise exposure of the ribosyl-phosphate moiety and minimise exposure of the base itself. The site of attachment to the base had a profound effect on immune response, with some immunogens yielding little or no response. The UDP and PAP antibodies used in these experiments are polyclonals raised in rabbits, whereas the ADP antibody is a monoclonal. However, similar results were observed with a polyclonal ADP antibody, and in general the authors have not observed significant increases in antibody selectivity on transitioning to monoclonals. Interestingly, the fluor component of the tracer can have a profound effect – as much as 10-fold – on the selectivity of the antibody in competitive binding assays.

The total time required for development of Transcreener™ detection reagents ranges from four to eight months, depending on whether a polyclonal or monocolonal antibody is used. Once the reagents are available for a family of group transfer enzymes, no further assay development is required other than buffer optimisation for specific enzyme isoforms or adjustment of antibody concentration to tune the assay sensitivity. This stands in contrast to most of the other group transfer assay methods (see Section 2), where new detection reagents such as antibodies or fluorescent probes must be developed for individual enzymes or subgroups of enzymes within a family.

The universality of the Transcreener™ platform is illustrated in **Figure** 4, where four different protein kinases were detected, each with a different acceptor substrate, using the same detection reagents. Note that one of the acceptors used was a protein, histone H1, instead of the more typical peptide. The ability to use native protein acceptor substrates is critical for kinases, such as Raf-1, where no peptide substrates have been identified. In addition, investigators have recently initiated efforts to develop kinase inhibitor drugs that bind at allosteric sites. It would be optimal to determine the binding properties and effects of such an allosteric inhibitor using a native protein acceptor; different effects might be observed with a non-physiological peptide substrate.

4. Expert opinion and conclusion

4.1 A single platform for kinase screening

Kinases are a validated target for anticancer drugs, and are rapidly being validated for other therapeutic areas as well including inflammatory diseases, diabetes and hypertension. There are several commercial kinase assay methods already available, each with their particular strengths and weaknesses but there is still real need for a more flexible HTS platform to accelerate the screening of new targets and for selectivity profiling across diverse family members. Transcreener™ is a non-radioactive generic kinase assay platform with the flexibility to

accommodate any protein kinase and any acceptor substrate. The availability of such a platform should simplify selectivity profiling and accelerate the screening of 'difficult' kinases that require native protein substrates. Moreover, by providing a single method and data output for all primary and secondary kinase screening functions the Transcreener™ platform has the potential to improve the overall kinase drug development process. Use of the same assay method for primary screening and selectivity profiling will enable a more integrated and iterative approach for the development of highly selective inhibitors, hopefully leading to more efficacious drugs with fewer side effects.

4.2 Assays for screening emerging target families

The involvement of glycosyltransferases and sulfotransferases in disease pathways is emerging and attracting the attention of pharmaceutical investigators searching for ways to develop more selective drugs. Transcreener™ is the only HTS assay method that is applicable to more than a very few enzymes in these emerging target families; as such it offers a tool for the rapid identification of chemical probes for target validation and of potential drug scaffolds for these novel families.

4.3 Elucidating the pharmacogenomics of conjugative drug metabolism

There is a critical need for robust *in vitro* screening assays to profile compound interaction with conjugative drug metabolising enzymes if the promise of pharmacogenomics is to be fully realised. Adverse reactions caused by drug interactions, improper dosing, and other drug metabolism-related events cause almost half of all drug candidate failures [65,66], and the link between genetic variation in drug metabolising enzymes and differences in drug response is widely recognised [67,68]. Individual variation in drug response could be managed more effectively if drug developers knew a) which enzyme(s) were catalysing metabolism of drug candidates, and b) how genetic variation affects enzyme selectivity and kinetics [69]. The key missing piece is *in vitro* screening assays for profiling compound interaction with individual drug metabolising enzymes and their allozymes during preclinical development. Although

molecular assays for CYP450-dependent drug oxidation are available and have been widely adapted in most drug discovery programmes [25], methods for assessing conjugative drug metabolism have lagged behind. Because the method allows direct screening for acceptor substrates, Transcreener™ assays for glycosylation and sulfation will provide the molecular basis for a comprehensive pharmacogenomic approach for the many diseases where these conjugative reactions plays a role in the metabolism of therapeutics, the disease pathways, or both.

4.4 Mapping cellular group transfer reactions

At a more fundamental level, the ability to screen directly for acceptor substrates with Transcreener™ will allow delineation of the endogenous roles for the many group transfer enzymes whose functions are not well understood. Despite the intense focus on protein kinases over the past 20 years, knowledge of physiological phosphorylation sites is far from complete. Elucidation of endogenous acceptors lags even further behind for the group transfer families that modify and regulate small molecules, such as sulfotransferases, glycosyltransferases and methyltransferases. It is abundantly clear that covalent modification of hormones and other small signalling molecules is extensive and functionally relevant. Examples involving sulfation listed above (**Table 3**) include regulation of the stability and activity of steroid hormones [51], and transcriptional control of cholesterol biosynthetic enzymes by cholesterol sulfates acting as nuclear receptor ligands [49]. The same family of 11 cytosolic sulfotransferases is involved in the metabolism of many commonly prescribed drugs, including chloramphenicol, acetaminophen and tamoxifen [36]. How frequently do drug–hormone interactions occur at sulfotransferases enzymes, and what are their consequences for patients? What is the potential of hormone modifying group transfer enzymes as drug targets? (entacapone is a clinically approved catechol methyltransferase inhibitor that is used in combination with L-DOPA to treat Parkinson's disease [70]). Hopefully, flexible assay platforms like Transcreener™ will begin to provide answers to these questions, leading to a more physiologically integrated approach to drug discovery and decreased potential for adverse side effects.

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