Viikki Drug Discovery Technology Center
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# Screening Methods for the Evaluation of Biological Activity in Drug Discovery

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ACADEMIC DISSERTATION

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## ABSTRACT

The design and development of new screening assays in drug discovery has become increasingly important during the past few years. Demands for high productivity have increased the need to screen more targets with higher throughput and more information content in combination with lower costs. To be able to meet these demands, a great deal of research is required in areas such as target selection and in the development of improved methodologies for detection and cell-based screens.

Miniaturised screening assays, both biochemical and cell-based, were developed and optimised for the evaluation of biological activity against diverse targets, including protein kinase C, voltage-gated calcium channels, *Chlamydia pneumoniae* and Caco-2 cells. Particular emphasis was placed on assessment of assay quality and validation in which quality parameters such as signal-to-background (S/B) and signal-to-noise (S/N) ratios, and screening window coefficient (Z') were employed. A compound library containing natural compounds and their derivatives, antimicrobial agents as well as other known medical substances was used as a source for the model compounds needed for screening and method validation.

Biochemical assays, a kinase activity assay and [$^3$H]-phorbol ester binding assay were used for primary screening of a set of compounds to establish their potential inhibitory effects on protein kinase C (PKC). The most potent inhibitors of kinase activity were (-)-epigallocatechin gallate and (-)-epicatechin gallate. In addition, dodecyl gallate, and the flavonoids myricetin, quercetin, rhamnetin, luteolin, isorhamnetin and kaempferol also had significant effects on PKC activity. However, no marked inhibition of the binding of phorbol ester to the regulatory domain of PKCα was observed, suggesting that the mechanism of PKC inhibition could be the result of binding to the catalytic domain of PKC. The suitability of these PKC assays for screening was demonstrated by the excellent values obtained for assay repeatability, reproducibility and quality parameters.

A $^{45}Ca^{2+}$ uptake assay based on clonal rat pituitary cell line $GH_4C_1$, possessing L-type voltage-operated calcium channels, was miniaturised into a 96-well plate format and optimised to improve performance and cost effectiveness. The validity of the assay was demonstrated by the fact that the results were consistent with previous data from Petri dish assays and by the high assay quality. Miniaturisation resulted in considerable savings in time, labour and resources, and the suitability of the assay for automation was demonstrated on a Biomek FX workstation, thus further improving the applicability of this assay for screening programmes. The automated $^{45}Ca^{2+}$ uptake assay was also successfully coupled with HPLC micro-fractionation for primary detection of calcium antagonistic components in complex matrices shown with a root extract of *Peucedanum palustre*, significantly reducing the time needed for bioactivity-guided isolation of active compounds.

A novel time-resolved fluorometric immunoassay (TR-FIA) was developed and validated for susceptibility testing of *Chlamydia pneumoniae*. By constructing a europium-labelled antibody we were able to design a cell-based, 96-well plate assay where chlamydial inclusions can be quantified as time-resolved fluorometric signals by means of a multilabel counter. Minimum inhibitory concentrations (MIC) measured using the TR-FIA demonstrated good to excellent correlation with those of two reference methods, immunofluorescence staining and real-time PCR. TR-FIA also offers the possibility for simultaneous cytotoxicity assessment by means of dual labelling with a protein dye, sulphorhodamine B. This novel assay significantly facilitates the laborious

methodology needed for the detection of intracellular bacteria and eliminates the subjectivity of traditional staining methods.

The ability of compounds to interact with biomembranes and cellular permeability is of great importance during the screening process. In this study, a set of flavonoids and alkyl gallates were studied using transport studies on Caco-2 cells and membrane affinity experiments in phospholipid vesicles. The apparent permeability coefficients ($P_{app}$) from the Caco-2 and the partition coefficients ($K_d$) from the membrane affinity experiments yielded similar information on the biomembrane interactions of flavonoids, showing that strong membrane affinity was generally accompanied by poor apical to basolateral transport in Caco-2 cells. Therefore, in this case the biochemical membrane affinity assay would have been a good predictor of a compound's permeability characteristics in Caco-2 cells, and could be a useful, less laborious tool for eliminating compounds with undesirable permeability characteristics from large compound pools.

In conclusion, the design of a screening assay is an array of multiple choices, all of which have significant impacts on the outcome of the overall drug discovery process. Most importantly, the correct selection of the target and assay format, detailed optimisation and miniaturisation as well as the choice of appropriate detection technology for each individual assay can lead to savings in time, money and labour along with improved data quality in all stages of the drug discovery process. The knowledge gained in this study on assay development, miniaturisation and automation has provided important and useful information for setting up bioactivity screening programmes in academic settings.

## LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications referred to in the text by the Roman numerals I-V.

**I** Tammela, P., Ekokoski, E., García-Horsman, A., Talman, V., Finel, M., Tuominen, R., Vuorela, P., Screening of natural compounds and their derivatives as potential protein kinase C inhibitors. *Drug Dev. Res.* (2004), in press.

**II** Tammela, P., Vuorela, P., Miniaturisation and validation of a cell-based assay for screening of $Ca^{2+}$ channel modulators. *J. Biochem. Biophys. Methods*. (2004) 59, 229-239.

**III** Tammela, P., Wennberg, T., Vuorela, H., Vuorela, P., HPLC micro-fractionation coupled to a cell-based assay for automated on-line primary screening of calcium antagonistic components in plant extracts. *Anal. Bioanal. Chem.* (2004) 380, 614-618.

**IV** Tammela, P., Alvesalo, J., Riihimäki, L., Airenne, S., Leinonen, M., Hurskainen, P., Enkvist, K., Vuorela, P., Development and validation of a time-resolved fluorometric immunoassay for screening of antichlamydial activity using a genus-specific europium-conjugated antibody. *Anal. Biochem.* (2004) 333, 39-48.

**V** Tammela, P., Laitinen, L., Galkin, A., Wennberg, T., Heczko, R., Vuorela, H., Slotte, J.P., Vuorela, P., Permeability characteristics and membrane affinity of flavonoids and alkyl gallates in Caco-2 cells and in phospholipid vesicles. *Arch. Biochem. Biophys.* (2004) 425, 193-199.

## ABBREVIATIONS

| | |
|---|---|
| A | absorption of photons |
| ADME | absorption, distribution, metabolism, excretion |
| ADMET | absorption, distribution, metabolism, excretion, toxicology |
| ADP | adenosine diphosphate |
| ATCC | American Type Culture Collection |
| ATP | adenosine triphosphate |
| BRET | bioluminescence resonance energy transfer |
| Caco-2 | human colon adenocarcinoma cell line |
| cAMP | cyclic 3',5'-adenosine monophosphate |
| CNS | central nervous system |
| 3D | 3-dimensional |
| DAD | diode array detector |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| DTPA | diethylenetriaminepentaacetic acid |
| DTTA | diethylenetriaminetetraacetic acid |
| EB | elementary body |
| EMEA | European Agency for the Evaluation of Medicinal Products |
| Eu | europium |
| F | fluorescence |
| FCS | fluorescence correlation spectroscopy |
| FDA | US Food and Drug Administration |
| FI | fluorescence intensity |
| FIDA | fluorescence intensity distribution analysis |
| FL | fluorescence lifetime |
| FLIM | fluorescence lifetime imaging microscopy |
| FP | fluorescence polarisation |
| FRET | fluorescence resonance energy transfer |
| GFP | green fluorescent protein |
| $GH_4C_1$ | clonal rat pituitary gland cell line |
| GPCR | G protein-coupled receptor |
| HBSS | Hanks' balanced salt solution |
| HCS | high-content screening |
| HIV | human immunodeficiency virus |
| HL | cell line originating from the human respiratory tract |
| HPLC | high-performance liquid chromatography |
| HRS | high-resolution screening |
| HTS | high-throughput screening |
| IAM | immobilized artificial membrane |
| IC | internal conversion |
| $IC_{50}$ | concentration yielding 50% inhibition |
| IF | immunofluorescence |
| IFU | inclusion-forming unit |
| ISC | intersystem crossing |
| $K_d$ | partition coefficient |
| LC | liquid chromatography |

| | |
|---|---|
| LDH | lactate dehydrogenase |
| *luc* | luciferase gene |
| MCH | melanin-concentrating hormone |
| MIC | minimum inhibitory concentration |
| MS | mass spectrometry |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium |
| NAD | nicotinamide adenine dinucleotide |
| NCE | new chemical entity |
| P | phosphorescence |
| $P_{app}$ | apparent permeability coefficient |
| PAMPA | parallel artificial membrane permeation assay |
| PCR | polymerase chain reaction |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PKCα | protein kinase C isoform α |
| PMA | phorbol 12-myristate 13-acetate |
| POPC | 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine |
| RNA | ribonucleic acid |
| S | singlet state |
| SAR | structure-activity relationship |
| S/B | signal-to-background |
| Sf9 | insect cell line derived from *Spodoptera frugiperda* |
| S/N | signal-to-noise |
| SPA | scintillation proximity assay |
| SRB | sulphorhodamine B |
| T | triplet state |
| TEER | transepithelial electrical resistance |
| TRET | time-resolved energy transfer |
| TRF | time-resolved fluorometry |
| TR-FIA | time-resolved fluorometric immunoassay |
| uHTS | ultra-high-throughput screening |
| UV | ultraviolet |
| Vis | visible |
| VOCC | voltage-gated calcium channel |
| Z | screening window coefficient calculated from library sample data |
| Z’ | screening window coefficient calculated from control sample data |

# 1. INTRODUCTION

The discovery of a new drug is a long and expensive process. From the synthesis of a compound to its approval can take 10-20 years, with an estimated average of about 9-12 years (Dickson and Gagnon 2004). A recent survey of 68 randomly selected new drugs yielded a total pre-approval cost estimate of 802 million US dollars for the research and development costs when unsuccessful projects were also included (DiMasi et al. 2003). Considering the fact that the preclinical costs per approved new drug account for more than 40% of the total costs and yet only 21.5% of new chemical entities (NCE, a new therapeutic molecule or compound that has not been tested on humans) that begin Phase I human trials are eventually brought to market, optimisation in the early stages of drug discovery would benefit both the industry and the consumer (DiMasi et al. 2003, Frank 2003, Ng and Ilag 2004).

For decades, bioactivity screening has been an integral part of the modern drug discovery process. The progress made in biochemistry, genomic sciences, combinatorial chemistry, etc. gave rise to the establishment of high-throughput screening (HTS) technologies (Drews 2000). The demand for higher productivity has increased the need to screen more targets with higher throughput and lower costs. From the mid-1990's major investments have been made in automated systems and novel technologies, and these have led to significant improvements in the speed, capacity and costs of HTS programmes. At the beginning of the 1990s, the number of data points generated by large screening programmes at a pharmaceutical company amounted to roughly 200,000 and rose to 5 to 6 million by the mid-decade (Drews 2000). Currently, in typical ultra-high-throughput screening (uHTS) campaigns 500,000 to 2 million compounds can be screened in 1-3 weeks with more than 100 targets per year.

Despite the advances and investments in drug discovery technologies during the past decade, there has been no improvement in productivity in terms of the approval of NCEs (Schmid and Smith 2004). The number of new products reaching the market is still declining: in 2002, the US Food and Drug Administration (FDA) approved only 18 new molecular entities and the European Agency for the Evaluation of Medicinal Products (EMEA) only 13 new medicinal products (Frantz and Smith 2003). It has been argued that too much attention has been focused on the number of compounds that could be assayed using HTS, with insufficient critical evaluation of the quality of

the data generated, particularly in regard to interaction specificity and predictive absorption, distribution, metabolism, excretion and toxicology (ADMET) (Cooper 2003).

The unfulfilled expectations placed on HTS and the high failure rate of discovered NCEs are probably the main driving forces behind the current industry-wide shift towards cell-based screening, which produces biologically more relevant information starting from the early stages of drug discovery (Johnston and Johnston 2002). It is noteworthy that many of the successful drugs on the market today were identified 30-40 years ago using traditional pharmacological approaches, i.e. functional cell-based assays, which can be seen as an advocate for the change towards using cellular assays in HTS (Moore and Rees 2001). Due to recent advances in assay technology, instrumentation and automation, the use of mammalian cell-based assays has expanded to all stages of the lead generation process, including primary screening. Increasing attention has been given to assay design and quality in all aspects of bioactivity screening such as target identification and validation, detection technologies and assay automation. Novel screening approaches tend to move towards high-content screening (HCS) through in-depth use of bioinformatics, computational methods for the filtering and focusing of compound libraries, and automated fluorescence microscopy imaging (Entzeroth 2003, Walters and Namchuk 2003). The definitive goals in modern HTS are lower cost and higher information content, leading from hits to high-quality lead compounds.

# 2. REVIEW OF THE LITERATURE

## 2.1. The process of generating lead compounds

The drug development process has been described as a multi-stage, multi-period set of choices (DiMasi et al. 2003). The lead generation alone consists of diverse sectors that require interdisciplinary experience, i.e. extensive knowledge in the fields of pharmacology, biochemistry, molecular biology, chemistry, engineering, etc. (Fig. 1). The initial step in drug discovery involves the identification and validation of a target, commonly a disease-linked protein, usually stemming from the genome, modulation of which is predicted to produce the desired effect. The design and development of a screening assay for the evaluation of activity is of the utmost importance in the process. The selected assay should be designed to examine the target activity with high sensitivity and specificity in a setting that mimics the *in vivo* dynamics as far as possible. The decisions made in assay design are governed by several factors, including the nature of the target and the pharmacological information sought. The resulting assay and data quality is significantly affected by the choice made between the different available detection technologies and assay formats. The implementation of HTS strategies, including miniaturisation and adequate automation, sets further criteria for the assay. The identification of hit candidates generally involves screening of collections of compounds or natural products, and hits enter an array of secondary screens, typically designed to evaluate the biological activity in more complex assay systems to confirm the activity observed in primary screening. The preclinical follow-up evaluation of hits includes analysis of compound efficacy and pharmacology, and studies of toxicology, specificity, biopharmaceutical properties and drug interactions from many aspects. Based on these criteria, hit compounds are selected for optimisation by synthetic chemistry and for more extensive preclinical evaluation in progressively more complex systems, from cells to whole organisms. In the lead optimisation phase, the hit molecule obtained from HTS is subjected to methodological synthetic modification by medicinal chemists in order to optimise the compound characteristics, including its activity and ADMET profile. The preclinical data forms the basis for the pharmacology section of an investigational new drug application to carry out clinical trials. (Lipsky and Sharp 2001, Bleicher et al. 2003, Dove 2003, Kenakin 2003, Knowles and Gromo 2003, Verkman 2004)

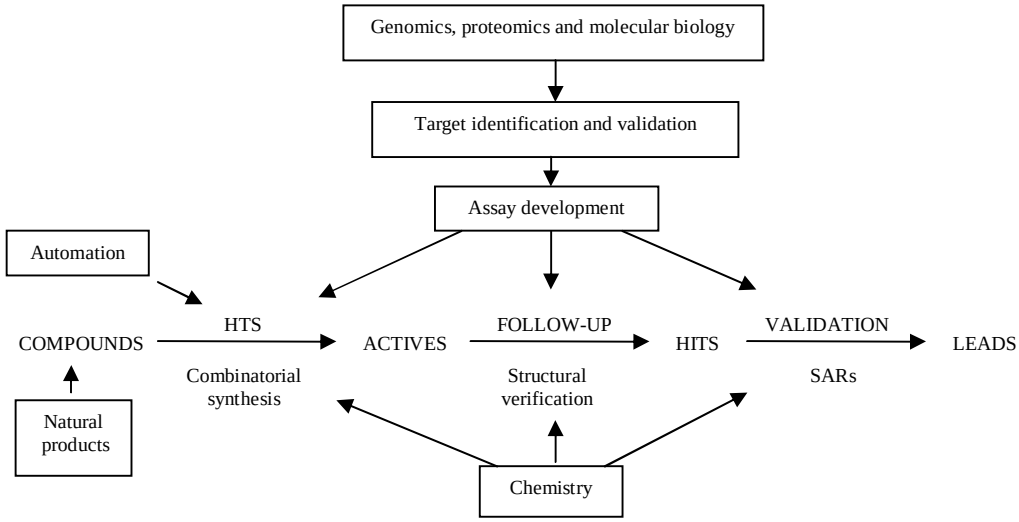


**Figure 1.** The lead generation process (SARs = structure-activity relationships) (adapted from Johnston and Johnston 2002).

## 2.2. Target identification and validation

The basis for screening assay development is a good, validated target. It has been estimated that 30-40% of experimental drugs fail because an inappropriate biological target was pursued (Butcher 2003). In target validation, the ultimate objective is to establish a crucial role for the molecular target in question in the cause or symptoms of a human disease (Williams 2003). To be judged as validated, genetic or pharmacological manipulation of a target should consistently lead to phenotypic changes that are in line with the desired effect in a dose-dependent manner. The desired changes must also be inducible in at least one relevant animal model, reflecting aspects of the human pathogenesis of the respective disease. In addition, the way in which the manipulation of a target molecule leads to a particular phenotype should be recognised, and whether or not other gene products are involved, with the possible attendant danger of toxic side effects (Drews 2003).

The recent publication of the human genome (Lander et al. 2001, Venter et al. 2001) revealed the presence of 30,000-40,000 genes on human chromosomes leading to an overabundance of possible drug targets. The present number of known and well-validated drug targets is, however, relatively

small. About 3,000 genes of the human genome belong to so-called drug-target families, but only a small minority of these genes are related to targets that are actually affected by pharmaceutical compounds. According to recent estimates, only less than 500 drug targets are involved in the mechanism of action of currently known drugs (Drews 1996, Drews 2000, van Duin et al. 2003). The number may actually be even smaller: a recent review identified only 120 targets for all marketed drugs (Hopkins and Groom 2002). However, it has been estimated that there are 500,000 different proteins, or potential points of pharmacological intervention, in the human body (Davies et al. 2004).

Selecting a target can be approached from several different angles. The gene pool can be filtered by concentrating on the prime candidate gene families encoding G protein-coupled receptors (GPCR), nuclear receptors, ion channels and kinases or on the orphan targets of these families (Butcher 2003, van Duin et al. 2003). Novel targets can be pursued by using genotyping of patient tissues or by manipulating the activity of the potential target protein itself using a proteomics approach (Sundberg et al. 2000). Another way to target validation is to disrupt the gene expression to reduce the amount of the corresponding protein. This can be achieved using antisense technology, ribonucleic acid (RNA) interference and genetic knockouts (Harris 2001, Butcher 2003, Smith 2003, Williams 2003). In the chemical genomics approach, the function of an unknown target can be defined by finding high-affinity binders to that target to determine what effects the binding has on intact cellular systems (Williams 2003). Recent progress in drug discovery has seen a realisation of the limitations of genomics, proteomics or other approaches *per se*, and the focus has shifted to using a unified approach to address the different aspects of target validation, e.g. by combining protein-interaction mapping of biological pathways with corresponding target information of known small-molecule compounds showing efficacy (Brown and Superti-Fuga 2003, Ng and Ilage 2004) or by using a multiple orthogonal tools approach where several tools are combined so that the target and the candidate therapeutic agent can be varied orthogonally independently of each other (Fig. 2) (Hardy and Peet 2004). However, despite all the efforts made in the early stages of drug discovery, target validation is only conclusive when the drug has finally been shown to be effective in the targeted human disease (Williams 2003).

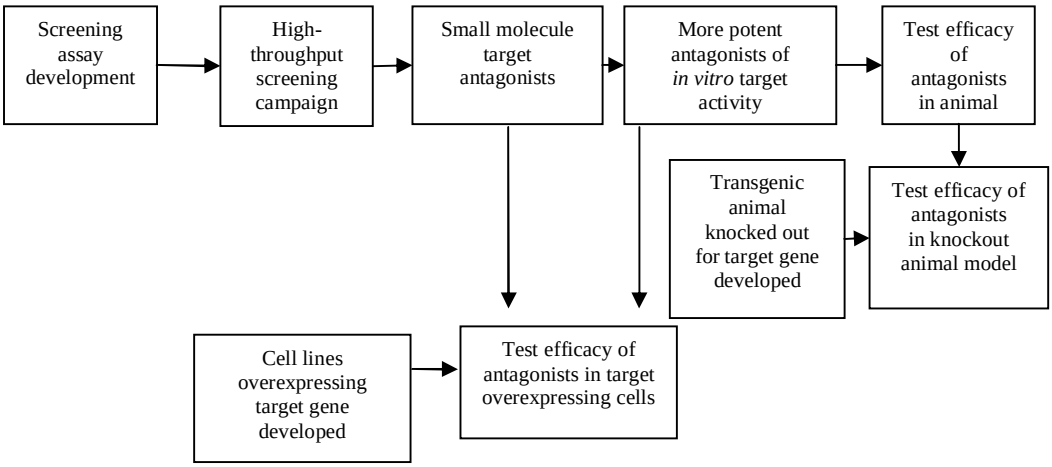


**Figure 2.** The multiple orthogonal tools approach in target validation (modified from Hardy and Peet 2004).

## 2.3. Sources for lead compound discovery

Novel medicinal lead compounds are sought by assaying large compound collections. When these screening libraries are being designed structural diversity is the main principle pursued (Nilakantan and Nunn 2003). Compounds for the libraries can be created by combinatorial chemistry or searched from natural sources. Focused libraries can be constructed by combining combinatorial chemistry with structure-based drug design, which involves the use of the 3-dimensional (3D) structure of a target protein, or a theoretical model obtained from homology modelling, to study the interactions between potential lead compounds and the target. However, the diversity offered by compounds produced by combinatorial chemistry is limited compared to compounds derived from natural products (Strohl 2003). According to Newman et al. (2003), during the period 1981-2002 no *de novo* combinatorial compound approved as a drug could be identified. In fact, the power of combinatorial chemistry lies mostly in optimising compound characteristics such as absorption, distribution, metabolism and excretion (ADME) profile, in all phases of drug discovery.

During the 1990's the progress made in developing techniques for combinatorial chemistry led to diminished interest in natural products, which were considered unsuitable for modern HTS

(Simmonds 2003), and the use of natural products as sources of new leads was widely disregarded. Yet, for decades the majority of drugs have been discovered from natural products: 61% of the 877 small-molecule new chemical entities introduced as drugs worldwide during 1981-2002 can be traced to, or were inspired by, natural products (Newman et al. 2003). Additionally, biologically active natural products are generally small molecules with drug-like properties, capable of being absorbed and metabolised by the body (Harvey 2000). Lately this potential has again been realised in screening programmes, which has led to the increased exploitation of natural sources. In fact, strategies have been described for the overall process of finding active components from nature (Vuorela et al. 2004).

Interest in screening natural sources for new leads has also grown because certain practical difficulties in separation technologies and in the speed and sensitivity of structure elucidation have been overcome in the case of natural products (Hostettmann et al. 2001). Active components can now be identified direct from complex mixtures such as extracts employing a high-resolution screening (HRS) approach, which includes chromatographic separation followed by on-line biochemical detection parallel with chemical characterisation (Schenk et al. 2003). As an example, Danz et al. 2001 have been able to identify on-line the cyclooxygenase-2 inhibitory principle in *Isatis tinctoria* using a hyphenated technique of liquid chromatography (LC) with a diode array detector (DAD) and mass spectrometry (MS) in combination with a microtitre-based bioassay. On the other hand, high-throughput techniques developed for combinatorial chemistry can be utilised to generate large libraries of purified fractions of small-molecule natural products, further enabling the reintroduction of natural products as an important source for drug discovery (Eldridge et al. 2002).

Historically, the mostly exploited natural source has undoubtedly been the plant kingdom, which still offers major opportunities as less than 5% of existing plant species have been chemically examined so far. The second most successful natural source has been fungi, from which breakthroughs like antibiotics, immunosuppressants and anticancer drugs have been discovered. Recently, much attention has also been given to less conventional natural sources including organisms of marine origin (Harvey 2000, Tulp and Bohlin 2004).

## 2.4. Assay design and development

The conversion of a biological target into a screening assay requires not only an understanding of the biology and biochemistry underlying both the disease and the readout of the assay but also an understanding of the other components involved in HTS, including automation strategies (Bronson et al. 2001). Assay design is based on the chosen target and depends mostly on the nature of the target and on the type of information sought, but also on the availability of reagents and plate readers, on the adaptability to miniaturisation and automation as well as on the stage of the project in the drug discovery process (Moore and Rees 2001, Seethala 2001). One of the most important issues in developing a cost-effective HTS method is the low cost and easy production of the target. Assay formats employed in screening can be basically divided into two main categories: cell-based and biochemical, i.e. isolated target screens (enzymes, receptors, etc.). Both formats have undoubted value, and usually a number of factors influence the choice of format (Johnston and Johnston 2002). One of these factors is the size of the library to be screened, i.e. uHTS programmes are typically based on biochemical screens. Basically, to be suitable for HTS, an assay must be robust, reproducible, and automatable (Bronson et al. 2001).

The current trend in drug discovery is clearly shifting towards cell-based assays starting from the primary screening stage. The advantages of cell-based screening are multiple. Most importantly, cell-based functional assays can provide biologically more relevant information on the compound's activity at a receptor or ion channel, and on the nature of this activity, information that cannot be achieved from a biochemical assay. Some targets may not be adequately configured in a biochemical assay due, for instance, to complex interactions between receptors and other cellular elements (Moore and Rees 2001, Johnston and Johnston 2002). In addition, cellular assays can simultaneously yield information on compound cytotoxicity and cellular membrane permeability. In fact, pharmaceutical companies have also started to screen drug candidates as early as possible for predictive *in vitro* indicators of *in vivo* activity and bioavailability, e.g. permeability in human colon adenocarcinoma (Caco-2) cell culture model, to reduce the number of compounds advancing to the traditional, more time-consuming secondary assays (Withington 2002, Cooper 2003). With most cell-based screens the logistics are more challenging than with biochemical screens, and significant assay development and investments in cell culture infrastructure are required (Moore and Rees 2001). Of importance is also the selection of cell line to be used, preferably with stable,

high-level expression of the target. In most cases, cellular assays are not yet amenable to uHTS (Boguslavsky 2004b). The compound's cytotoxicity can also mask its activity at the target, the result being false positives or negatives (Johnston and Johnston 2002).

The pursuit of cost savings through assay miniaturisation has resulted in the development and increasing use of homogeneous assay formats (so-called 'mix and measure' assays) which require only a series of additions without any separation steps (Bosse et al. 2000). Homogeneous assays enable the use of high-density well formats reducing the requirements set for performing the assay, e.g. by using automated liquid handling. Most homogeneous assays involve isolated targets and are fluorescence-based, but cellular assays have also been recently employed in homogeneous format, e.g. reporter assays based on green fluorescent protein (GFP) (Seethala et al. 2001).

Lately, approaches have evolved to increase the data content and quality from HTS. The term 'high-content screening' (HCS) summarises cellular assays based on sub-cellular imaging and automated image analysis (Gribbon and Sewing 2003). Work is also being done to obtain multiple data points from a single well of a plate by employing multiplexed and multi-parametric assay formats. The former are assays that produce a single measurement for each multiple cell type within a single well, and the latter yield multiple measurements from a single cell type (Beske and Goldbard 2002). An assay measuring multiple parameters has been described for instance for the evaluation of apoptosis by Lövborg et al. (2004) who were able to measure simultaneously the caspase-3 activity using fluorescein-tagged probes, mitochondrial membrane potential by chloromethyl-X-rosamine and nuclear morphology by deoxyribonucleic acid (DNA)-binding dye. A multi-parametric homogeneous time-resolved fluorescence quenching-based assay capable of detecting the activity of three caspases in a single well by using chelates of europium, samarium and terbium has also been reported (Karvinen et al. 2004).

Multiplexing cell-based assays is providing new potential for cell-based screening by enabling investigation of multiple targets simultaneously and the possibility of including controls within each well (Martins 2002). Obviously, certain limitations are involved, e.g. the need for similar culture requirements for the cell lines and possible cellular cross-talk (Beske and Goldbard 2002). Multiplexing has been successfully exploited in studying the agonists of nuclear receptors (Grover et al. 2003) as well as in developing an HTS platform for protein-protein interactions using a luciferase-based assay (Nieuwenhuijsen et al. 2003). Thus, in addition to increasing assay

throughput by incorporating low volume, high-density formats, further improvements can be made through the use of multiplexing and multi-parametric strategies.

Once a suitable assay format and design have been chosen, detailed refinement is needed to ensure that the assay is robust and consistent throughout the screening programme. In enzymatic assays, optimisation and validation for HTS involves steps addressing reaction conditions (buffers, pH, ionic strength, temperature), substrates (natural and synthetic), enzyme source and kinetics, estimation of signal windows and the responses of known inhibitors (Bronson et al. 2001). All reagents from plates and chemicals to biological material should be examined for lot-to-lot variability and stability. In cell-based assays, particular attention should be given to the characterisation of cellular growth under different conditions, and optimal cell seeding densities, cellular adherence to microplates, dimethylsulphoxide (DMSO) tolerance and within-in-plate variation in the signal during long-term incubations also need to be determined (Moore and Rees 2001, Johnston and Johnston 2002). Particularly in cell-based assays, the creation of precise operational procedures becomes necessary because of the sensitivity of most cell lines to alterations in properties due to small changes in environmental conditions. The signal reproducibility and the robustness of the signal window should also be examined, and this data is typically used to establish an active criterion for the HTS during assay development (Johnston and Johnston 2002). Following optimisation, an assay should be validated to ensure its consistency in the quantitative determination of active compounds (Bronson et al. 2001).

## 2.5. Detection technologies

Advances in technology during the past few years have led to the emergence of a variety of detection technologies for use in screening assays. In particular, fluorescence detection is more and more exploited as assay readouts, although methods based on radioactive isotopes are also still widely used. The trend towards the use of non-radioactive labels is the result of health and safety concerns, other factors being cost-effectiveness and the avoidance of generating radioactive waste (Bronson et al. 2001). However, problems are also related to the detection of fluorescence signals: the signal can be subject to quenching by compounds, plastics or media, fluorescence emissions can be scattered by particulates, or the signal can be masked by autofluorescence (from protein or compounds) or by high background fluorescence from unbound labelled probes (Rogers 1997). The

detection modalities typically utilized in HTS and drug discovery can be divided into four main categories: 1) fluorometric, 2) radiometric, 3) luminometric, and 4) photometric detections. The following chapters will give short descriptions of all these categories.

### 2.5.1. Fluorometric detection

Fluorescence occurs when photons of light absorbed by a molecule (in ~ $10^{-15}$ s) cause electrons to become excited to a higher electronic state and to return to the ground state after approx. $10^{-8}$ s with emission of light energy. The light emitted has a longer wavelength than that absorbed (the Stokes shift) as a result of a small dissipation of energy during the excited state (Lakowicz 1983, Emptage 2001). Fluorescence can be schematically illustrated by a Jablonski diagram (Fig. 3), first proposed by prof. A. Jablonski (1933) to describe the absorption and emission of light. Prior to excitation the electronic configuration of a molecule is defined as being the ground state. Once a molecule has been excited to a higher energy and vibrational state, there are a number of routes by which the electron can return to the ground state: if the photon emission occurs between the same electron spin states this is termed fluorescence, and if the spin states of the initial and final energy levels are different, the emission is called phosphorescence.

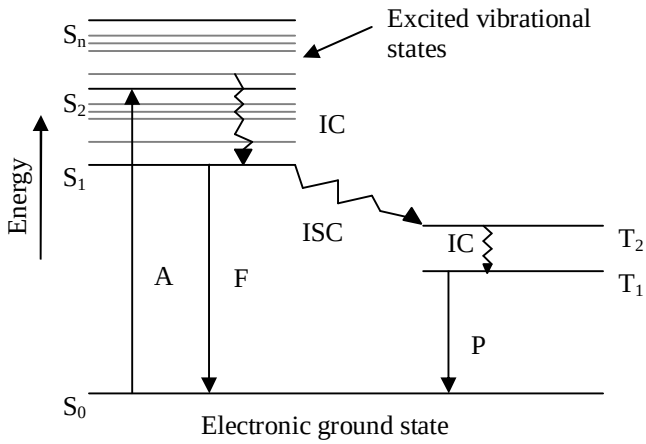


**Figure 3.** The Jablonski diagram. A = absorption of photons, F = fluorescence, P = phosphorescence, S = singlet state, T = triplet state, IC = internal conversion, ISC = intersystem crossing (modified from Jablonski 1933 and Lakowicz 1983).

In an attempt to improve the characteristics of fluorometric detection, multiple modifications have been created and used for diverse assay formats including homogeneous and cell-based assays. These modifications can be categorised as 1) fluorescence intensity (or prompt fluorescence, FI), 2) fluorescence polarisation/anisotropy (FP), 3) fluorescence resonance energy transfer (FRET), 4) fluorescence lifetime (FL), 5) time-resolved fluorescence (TRF) and 6) single-molecule detection methods, such as fluorescence intensity distribution analysis (FIDA) and fluorescence correlation spectroscopy (FCS) (Hemmilä and Hurskainen 2002, Gribbon and Sewing 2003).

***Fluorescence intensity (FI).*** The simplest form of fluorescence, FI, typically utilises fluorescent enzyme substrates and indicators loaded into membranes or compartments that alter their intensities due to environmental change (González and Negulescu 1998, Pope et al. 1999). The measured steady state FI is linearly related to changes in fluorophore concentrations. A well-known example of intensity-based indicators for cell-based assays is Fluo-3, a fluorescein-based $Ca^{2+}$ sensor whose FI increases approx. 100-fold upon binding $Ca^{2+}$ (Minta et al. 1989). Owing to the simple methodology, FI is widely used, although it is also very much affected by compound quenching, autofluorescence effects and inner-filter phenomena (Pope et al. 1999, Gribbon and Sewing 2003). Recently, the use of FI has been described in measuring the enzyme activity of several protein phosphatases (phosphoserine/phosphothreonine phosphatases and phosphotyrosine phosphatases), which are critical components in cellular regulation (Kupcho et al. 2004). This homogeneous application exploits fluorogenic peptide substrates (rhodamine 110, bis-phosphopeptide amide) that do not fluoresce in their conjugated form; however, upon dephosphorylation by the phosphatase of interest, the peptides become cleavable by the protease and release the highly fluorescent free rhodamine 110. In this work, FI was used as a detection method in the real-time polymerase chain reaction (PCR) of *Chlamydia pneumoniae* and in the microscopic visualisation of chlamydial inclusions by using a fluorescein isothiocyanate-labelled antibody **[IV]**.

***Fluorescence polarisation (FP).*** In FP experiments the excitation light is typically polarised and emission is measured in parallel and perpendicular orientations (Pope et al. 1999). The polarisation of the emitted light depends on how far the fluorophore rotates during the lifetime of its excited state. FP measures the rotation of single biomolecules or their complexes by interrogating the relative polarisation state of emitted fluorescence, i.e. the smaller the molecule, the faster it rotates, and the smaller its FP will be. Binding of a fluorescence-labelled ligand to its receptor in solution or on the surface of a living cell will result in slower rotation and an increase in FP (Rogers 1997,

Gribbon and Sewing 2003). FP is a ratiometric technique, and hence less prone to interference from inner-filter effects. It is also more tolerant to fluorescence quenching (for example by compounds, plastics, or media) and light scattering (Rogers 1997, Pope et al. 1999). Owing to the volume-independence of the polarisation signal and its rapidity due to homogeneous technology, FP is extensively used in HTS and several applications have been described. FP is typically exploited in assays to monitor hydrolytic or binding reactions (Pope et al. 1999). FP applications include a high-throughput screen to identify novel ligands of FK506 (immunosuppressive compound) binding protein 12 , which may have a role in neuronal survival (Bollini et al. 2002), as well as for the identification of inhibitors of heat shock protein 90, a molecular chaperone with essential functions in maintaining transformation (Kim et al. 2004).

***Fluorescence resonance energy transfer (FRET).*** Fluorophore pairs, or FRET pairs (donor and acceptor), with overlapping emission and excitation spectra are utilised in FRET. The excitation energy is transferred from a donor fluorophore to an acceptor via a dipole-dipole vectorial coupling when the acceptor is in close proximity to the donor (1-5 nm) (Wu and Brand 1994, Gribbon and Sewing 2003). In FRET experiments the ratio of donor and acceptor emission intensities is recorded, which makes the method minimally prone to variations in cell number, probe concentration or optical paths and results in improved reproducibility and signal-to-noise ratio. On the other hand, the spectral overlap between the emissions of donor and acceptor can reduce the dynamic range of the assay, and FRET changes can be limited by non-optimal fluorophore orientations or spacing (González and Negulescu 1998). FRET has been used for measuring the binding of inhibitors to stromelysin, which is involved in cancer, arthritis, restenosis, and other diseases that are caused by or result in the degradation of connective tissue matrices (Epps et al. 1999, Stetler-Stevenson et al. 1993). Recently, an HTS assay based on FRET has been described for screening of small-molecule inhibitors of prokaryotic ribosome assembly to identify lead compounds for potential antimicrobials (Klostermeier et al. 2004).

***Fluorescence lifetime (FL).*** The lifetime of a fluorescent probe, i.e. the average time the electrons spend in the excited state, can be used as a directly determined output parameter in HTS systems (Turconi et al. 2001, Gribbon and Sewing 2003). FL can be measured by illuminating the sample with a modulated continuous light wave, and the phase shift between the modulation of the excitation light and the emission of the fluorescence can be used to calculate the lifetime (frequency-domain FL). Alternatively the sample can be illuminated with a laser and the time-

dependent decay of the fluorescence intensity measured (time-domain FL) (Lakowicz 1983). FL is based on the intrinsic molecular property of the fluorophore that is not influenced significantly by light scattering in biological matter (Weber 1981, Lakowicz 1983, Wild 1977, Hovius et al. 2000). FL is able to detect minute changes in the fluorophore's immediate environment: a change in molecular brightness induced, for example, by a binding process results in a change in total fluorescence intensity and lifetime. FL is increasingly being used in HTS because of its sensitivity and robustness (Lakowicz 1983, Eggeling et al. 2003). However, changes in FL are not always predictable, and this limits the applicability of FL in biological reactions (Eggeling et al. 2003). To image molecular interactions in live cells, FL is exploited in fluorescence lifetime imaging microscopy (FLIM), which can provide information not only concerning the localisation of specific fluorophores, but also about the local fluorophore environment (Hovius et al. 2000). FLIM has been successfully exploited in studying the receptor tyrosine kinase activity in cells (Wouters et al. 1999), protein kinase C (PKC) regulation mechanisms (Ng et al. 1999), and to detect intermolecular FRET in living neurones (Duncan et al. 2004).

***Time-resolved fluorescence (TRF).*** TRF exploits fluorophores with long fluorescence lifetimes, such as rare earth elements like europium, samarium and terbium. As such, these lanthanides exhibit a very weak absorption of excitation energy and are poor fluorophores. In order to improve the collection of excitation energy, lanthanides are normally chelated with a ligand, e.g. diethylenetriaminetetraacetic acid (DTTA) or diethylenetriaminepentaacetic acid (DTPA), which is able to absorb excitation light and transfer the energy to the ion (Hemmilä and Harju 1994). The unique fluorescence properties of lanthanide chelates allow temporal resolution of the signal from the short-lived fluorescence caused by background interference (e.g. sample/reagent fluorescence, luminescence of plastics and optics), i.e. the signal can be measured once non-specific background fluorescence has decayed (Soini and Kojola 1983, Hemmilä et al. 1984). Other fluorescence properties of lanthanides, e.g. a substantial Stokes' shift (difference between excitation and emission peaks) and the narrow emission peak, contribute to increasing the signal-to-noise ratio. The sensitivity is further increased by the dissociation-enhancement principle, which means that the lanthanide is dissociated into a new highly fluorescent chelate inside a protective micelle. Lanthanide chelates can be used for protein, peptide and oligonucleotide labelling, and europium, which was first applied by Hemmilä and co-workers as a tool for immunofluorometric assays (Hemmilä et al. 1984), is frequently used for labelling antibodies. TRF is extensively employed in various applications, e.g. for studying leukocyte adhesion (Saarinen et al. 2000), for cytotoxicity

screening (Blomberg et al. 1986, Bouma et al. 1992), for measurement of interferon activity in relation to human cells (Trinh et al. 1999), and for identifying cyanovirin-N [a potent anti-human immunodeficiency virus (HIV) protein] mimetics among natural product extracts (McMahon et al. 2000). Applications in which TRF is used in combination with FRET (time-resolved energy transfer, TRET) have also emerged, examples being the identification of inhibitors of viral and bacterial helicases in 96- and 384-well plate formats (Earnshaw et al. 1999) and the detection of insulin receptor tyrosine kinase activity (Biazzo-Ashnault et al. 2001). In these experiments europium-labelled antibody has been employed as the donor and XL665-labelled streptavidin as the acceptor. In the present study, time-resolved fluorometric immunoassay exploiting europium-labelled antibody was developed for the evaluation of antichlamydial activity **[IV]**.

***Fluorescence intensity distribution analysis (FIDA) and fluorescence correlation spectroscopy (FCS).*** FIDA and FCS are based on the analysis of temporal fluctuations in the fluorescence signal detected from the spontaneous diffusion of individual fluorescent molecules into and out of a small, tightly focused confocal element. These techniques allow the determination of concentrations and specific brightness values of individual fluorescent species in a mixture (Ehrenberger and Rigler 1974, Auer et al. 1998, Kask et al.1999, Pope et al. 1999). In FCS, it is the diffusion characteristics and concentration of fluorescent molecules that are determined (Eigen 1994, Maiti et al. 1997), whereas in FIDA the amplitude information in the fluctuation signal is used to determine the fluorescence intensity and concentration of fluorescent molecules (Kask et al. 1999). These single-molecule detection methods can be applied for biochemical assays if the fluorescent analyte undergoes a change of brightness. The signal output in FIDA and FCS is independent of the total assay volume, making these techniques especially useful in HTS and uHTS (Pope et al. 1999). As examples, changes in fluorescence intensity measured by FIDA have been employed in measuring the enzyme activity of phosphotyrosine kinase, protease and alkaline phosphatase as well as the interactions of fluorescent-labelled ligands with a range of GPCRs, e.g. the binding of melanin-concentrating hormone (MCH) to MCH receptor (Rüdiger et al. 2001, Haupts et al. 2003).

### 2.5.2. Radiometric detection

In radiometric experiments, radioactive labels are employed to measure the biological activity of interest. These labels are usually produced by substituting an existing atom of a compound with its

radioisotope, e.g. $^{14}C$, $^{3}H$, $^{32}P$ or $^{125}I$. The presence of the radioactive compound can be measured by scintillation counting, in which the disintegration of the radioisotopic labels is quantified as particles emitted by the label (Aalto et al. 1994). The most important applications of radiometric measurement are in immunoassays, DNA hybridisation, receptor binding and cellular assays as well as metabolic studies (Hemmilä 1994). Radioactive labels have been gradually replaced by other labels, such as fluorescent and luminescent molecules, due to their disadvantages, which mostly relate to health and waste problems.

For HTS, however, a special assay format called scintillation proximity assay (SPA) that uses radiometric detection has been created (Udenfriend et al. 1987). SPA employs a scintillant embedded within a microbead matrix (or alternatively onto the surface of a microtitre plate) coated with a capture molecule (e.g. streptavidin or glutathione) to immobilise the target of interest. SPA provides an opportunity to measure ligand binding interactions (Bronson et al. 2001). When a radiolabelled ligand binds to its target on the surface of the bead, the radioactive decay occurs in close proximity to the bead. The energy is effectively transferred to the scintillant, resulting in the emission of light. When the radiolabel is displaced or inhibited from binding to the bead, it remains free in solution and is too distant from the scintillant for efficient energy transfer. Energy from radioactive decay is dissipated into the solution, which results in no light emission from the beads. Hence the bound and free radiolabel can be detected in homogeneous assay format without the physical separation required in filtration assays (Sittampalam et al. 1997b). Recently, applications exploiting SPA have studied the inhibition of cyclooxygenase-2 using a semi-homogeneous enzymatic assay for screening of plant secondary metabolites (Huss et al. 2002). In this study radiometric detection was employed in PKC assays **[I]**, for studying the uptake of $^{45}Ca^{2+}$ in clonal rat pituitary ($GH_4C_1$) cells **[II, III]**, and for the evaluation of monolayer integrity in Caco-2 cells by measuring the paracellular transport of [$^{14}C$]-mannitol **[V]**.

### 2.5.3. Luminometric detection

Detection techniques based on luminescence are well established and widely used in bioanalytical applications. These techniques are especially suitable for HTS because they allow rapid and sensitive detection of analytes and can be applied to small-volume samples (Roda et al. 2003). Luminescence can be categorised into three main types: 1) bioluminescence (biologically driven

conversion of chemical energy into light, involving photoproteins or enzymes that may be expressed within cells), 2) chemiluminescence (conversion of chemical energy into light by chemical reaction), and 3) electrochemiluminescence (electrochemically driven conversion of chemical energy into light, involves electrodes in contact with light-emitting chemicals in solution).

Luminometric techniques have been employed in both biochemical and cell-based assays. Bioluminescent and chemiluminescent enzyme activity assays are based either on the detection of the end products of the enzymatic reaction or on the direct, real-time evaluation of the rate of the enzyme-catalysed reaction through its coupling with a suitable luminescent system (Roda et al. 2003). Chemiluminometric detection has been used in protein kinase assays (Lehel et al. 1997) and in the screening of acetylcholinesterase inhibitors in 384-well format (Andreani et al. 2001). Bioluminometric assays utilising reporter genes such as luciferase (*luc*) and β-galactosidase offer a powerful tool for studying the ability of candidate drugs to interact with cellular pathways and the activation state of a receptor and are used to a large extent in HTS laboratories (Joyeux et al. 1997, Roda et al. 2003). Among reporter genes, the firefly *luc* is one of the most commonly used, and several transcription-based assays have been developed that allow the monitoring of GPCR activation (Naylor 1999).

The latest innovation in bioluminescent assay technologies is bioluminescence resonance energy transfer (BRET), a phenomenon similar to FRET occurring between a light-emitting luciferase donor and a fluorescent protein acceptor (Roda et al. 2003).

### 2.5.4. Photometric detection

In photometric detection the amount of light absorbed by a solution of an organic molecule (or the transparency of a suspension of bacterial cells) is recorded to gauge the concentration of the molecule present in the sample. This simple methodology makes photometric assays quite useful in certain systems, but their general lack of sensitivity limits their utilisation in miniaturised formats for HTS (González and Negulescu 1998). High concentrations of dyes and large cell populations are needed to achieve significant absorbance changes, and the methodology is prone to interference caused by an absorbing matrix. For this reason, in many systems photometric detection has been replaced by more sensitive methods such as fluorescence.

Assays based on ultraviolet/visible (UV/Vis) absorption have recently been used for enantioselective screening of nitrilase-producing microorganisms (Banerjee et al. 2003) and for studying the cytotoxicity of compounds using a variety of dyes including tetrazolium reagents to evaluate the viability of cells (Slater 2001, Mannerström et al. 2002, Riss and Moravec 2004). Photometric detection has also recently been employed in the identification of poly[adenosine diphosphate (ADP)-ribose] polymerase inhibitors by using a nicotinamide adenine dinucleotide (NAD)-based assay in a 96-well plate format (Brown and Marala 2002) as well as in the evaluation of angiogenesis activity in cultured rat aorta ring (Wang et al. 2004). In the current work, photometric detection was applied in cytotoxicity assays to measure the release of lactate dehydrogenase (LDH) from cells **[II]** and the cell proliferation and metabolic activity by using dyes such as sulphorhodamine B (SRB) **[IV]** and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) **[V]**.

## 2.6. Assay miniaturisation and automation

The need to screen an increasing number of compounds against a variety of therapeutic targets has resulted in technological advances in assay automation, miniaturisation and in the sensitivity and specificity of detection methods aiming to increase sample throughput and to decrease costs (Bosse et al. 2000, Sundberg 2000). High-throughput, low-volume assays have been created to achieve these goals, and today the plate formats available range from 96-well to up to 9600-well and even higher density formats and 'virtual wells' (Marron and Jayawickreme 2003). Significant savings can be achieved through miniaturisation; a cost-benefit analysis for miniaturising a protease assay for screening a library of 100,000 compounds is presented in Table 1.

**Table 1.** A cost-benefit analysis for miniaturising a protease assay for screening a 100,000-member compound library (adapted from Oldenburg et al. 2001).

| Well density | Well volume (µl) | Fold reagent savings | Total cost ($) |
|---|---|---|---|
| 96-well | 200 | 0 | 209,000 |
| 384-well | 50 | 4 | 139,500 |
| 1536-well | 1-10 | 20-200 | 56,100 |
| 6144-well | 0.2-0.7 | 250-1000 | 53,650 |

Assay miniaturisation is a process of establishing optimal assay conditions for the microlitre volumes necessary for micro-scale screening in high-density plate formats (Wölcke and Ullmann 2001). Despite the savings, miniaturisation is only feasible for robust assays with high, reproducible signals; for assays made with well densities above 1536-well formats the use of homogeneous assays is a necessity (Boguslavsky 2004a). Miniaturising an assay normally involves making several changes that are necessary for implementing a high-density plate format. Frequently, miniaturisation causes loss in assay performance (e.g. lower signal, increased variability, loss of robustness), resulting for example from increased exposure to oxygen, higher surface-to-volume interactions, and from the increased error associated with miniaturised liquid handling and signal detection (Taylor 2002). The use of miniaturised assay formats therefore requires new techniques and strategies for sample handling, assay development and assay adaptation. Automated plate and liquid handling with high accuracy are essential as well as refinement of the techniques used for detection and data handling (Wölcke and Ullmann 2001). In cellular assay systems, the degree of miniaturisation is usually limited due to biological variation and cell viability.

Depending on the throughput required, automation may need to be integrated into the assay development (Burbaum 1998, Hertzberg and Pope 2000, Elands 2001). Automation is usually considered to mean the use of workstations, robotic sample processors, plate stacking and moving devices, automated HTS/uHTS assay systems etc. when in fact laboratory automation has actually begun much earlier, starting from hand-held pipettors (Elands 2001). Microplate instrumentation can be classified into stand-alone devices (plate washers, dispensers, readers), workstations (e.g. liquid handlers) and robotic systems (a collection of stand-alone devices and workstations integrated into a functional environment, controlled by system management software and one or more robot arms to perform an application) (Elands 2001).

Before assays can be transferred onto automated platforms, improvements in robustness, stability, signal separation, and assay variability are often required (Taylor 2002). HTS requirements for automation include throughput that is not limited by robot, flexibility (easy set-up of new assays), and industrial-level reliability (long unattended runs, robust equipment, good support). Most important benefits and automation drivers are listed in Table 2. In addition to primary and secondary screening, robotics and other automated systems are currently used in various areas of drug discovery including early-ADME and toxicity studies (Trinka and Leichfried 2001). The

exponential growth in using cell-base assays in the pharmaceutical industry has also led to growing interest in cell culture automation for producing the cells needed for the assays (Koppal 2003).

**Table 2.** Benefits and drivers for assay automation.

| Benefits of automation | Automation drivers |
|---|---|
| unattended operation | demands for higher sample throughput |
| labour saving | flexibility to run different assay technologies |
| improved throughputs | increasing demand for laborious cell-based assays |
| improved results (accuracy, reproducibility etc.) | assay transfer to HTS should be fast and simple |
| operator-independence | demands for consistent, high quality data |
| environmental control | desire to focus screening personnel on assay development and data analysis |
| personnel safety (bio-/radiochemical) | |
| sample tracking and control | |

By employing homogeneous fluorescence-based assays and novel signal detection techniques, miniaturisation strategies, robotics and other laboratory automation it is possible to perform 100,000 assays per day, commonly affiliated with uHTS utilised in large-scale screening programmes in the pharmaceutical industry (Bronson et al. 2001). The implementation of uHTS further necessitates the development of specially designed data analysis and data management systems for scheduling of the screening devices, handling of compound data, management of screening results and for in-process quality control (Wölcke and Ullmann 2001).

## 2.7. Assay quality and validation

After initial development, the suitability of the assay for screening needs to be further evaluated and adequate validation should be performed to ensure the high quality of screening data. The shift from high-quantity screens towards higher information content and high-quality data has placed greater demands on the quality of assay performance. High quality in relation to an HTS assay can in principle be defined as the ability to separate the signals for inactive and active molecules sufficiently to allow accurate identification of hits (Sittampalam et al. 1997a, Zhang et al. 1999). Assays for HTS not only require small sample volume, high throughput and robustness, but also adequate sensitivity, repeatability and reproducibility (e.g. plate-to-plate and day-to-day variations), and accuracy in order to be suitable for automated liquid handling and signal detection

systems. Additionally, in most HTS programmes each compound is tested only singly or in duplicate, and high-quality data from the assay is therefore critical (Zhang et al. 1999). Equally, the assay signal should be able to avoid perturbation by the numerous non-specific effects that can originate from the assay components. Among the key assay interference factors are inner-filter effects, autofluorescence, quenching and photo-bleaching (Comley 2003).

The quality of an HTS assay is typically evaluated using quality parameters such as signal-to-background (S/B) and signal-to-noise (S/N) ratios and a screening window coefficient called the Z factor. The S/B ratio simply measures the intensity difference between the signal and background, whereas the S/N ratio also takes into account the impacts of signal and background variations. The Z factor is an indicator of assay quality and reflects more precisely both the dynamic range of the assay signal and the data variation associated with the signal measurements. The Z factor is the ratio of the separation band (i.e. signal window) to the dynamic range of the assay signal, and defines a parameter characterising the capability of hit identification for each given assay under the defined screening conditions (Zhang et al. 1999). The Z factor is calculated from data gathered from library samples showing no activity, whereas its more widely used modification, the Z' factor, is calculated using data from control samples. The following equations for the quality parameters have been described in Bollini et al. (2002) and in Zhang et al. (1999): $S/B = X_s/X_b$, $S/N = (X_s-X_b)/(SD_s^2 + SD_b^2)^{1/2}$, $Z' = 1- [(3 \times SD_s + 3 \times SD_b)/|X_s-X_b|]$. $X_s$ and $SD_s$ represent the average and standard deviation of the signal obtained from control samples exhibiting maximum signal. $X_b$ and $SD_b$ represent the average and standard deviation of the signal obtained from control samples exhibiting no specific signal (i.e. background).

As a dimensionless parameter, the Z factor is appropriate for evaluating overall assay quality and can be used in assay development and optimisation, e.g. to compare the effects of different assay conditions. The Z' factor is characteristic of the quality of the assay itself, without the intervention of test compounds. Basically, the higher the value of the Z or Z' factor of an assay, the higher the data quality. Screening assays can be categorised according to their respective Z factors, which enables different screening assays to be compared (Table 3).

**Table 3.** A simple categorisation of screening assay quality in terms of the value of the Z factor (Zhang et al. 1999, with modifications).

| Z factor value | Structure of assay | Related to screening |
|---|---|---|
| 1 | SD = 0 (no variation), or the dynamic range $\rightarrow \infty$ | An ideal assay |
| $1 > Z \geq 0.5$ | Separation band is large | An excellent assay |
| $0.5 > Z > 0$ | Separation band is small | A double assay |
| 0 | No separation band, the sample signal variation and control signal variation bands touch | A "yes/no" type assay |
| $< 0$ | No separation band, the sample signal variation and control signal variation bands overlap | Screening essentially impossible |

However, particularly in cell-based assays, the system is prone to avoidable variation, which needs to be taken into account when evaluating the assay quality. For cell-based assays, Z' factors > 0.4 can be accepted (Falconer et al. 2002).

# 3. AIMS OF THE STUDY

The process of finding high-quality drug candidates relies on robust, accurate and reproducible screening assays for the evaluation of biological activity of compound libraries. In this context, the overall aims of the study were to modify, develop and validate diverse methods in order to improve and demonstrate their suitability for screening, and to perform small-scale screening in an academic setting.

To achieve the overall aims the following investigations were conducted:

1) a set of natural compounds and their derivatives were screened against two enzymatic protein PKC assays exploiting kinase activity and [$^{3}$H]-phorbol ester binding, and the suitability of these assays for screening was investigated **[I]**
2) a cell-based $^{45}Ca^{2+}$ uptake assay was miniaturised, validated and automated and the applicability of the assay for detecting calcium antagonistic components of crude extracts in combination with high-performance liquid chromatography (HPLC) micro-fractionation was studied **[II, III]**
3) a novel time-resolved fluorometric immunoassay was developed for the evaluation of antichlamydial activity in order to increase the sample throughput, and the assay was validated with antimicrobial agents in comparison with two reference methods: immunofluorescence microscopy and real-time- PCR **[IV]**
4) the possibility of predicting the biomembrane interactions of compounds by membrane affinity experiments in phospholipid vesicles compared to transport studies in Caco-2 cells was studied using a set of flavonoids and alkyl gallates **[V]**
5) assay quality parameters were employed, their applicability evaluated, and the impacts of diverse assay formats, detection technologies and automation strategies on assay quality were assessed

# 4. MATERIALS AND METHODS

More detailed information on the experimental conditions can be found in the original publications **I-V**.

## 4.1. Model compounds

### 4.1.1. Standard compounds

The PKC inhibitor staurosporine (Sigma-Aldrich Co., USA) was employed as the positive control in the kinase activity assay, while phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Co., USA), competing with the [$^{3}$H]-phorbol ester, was used in the PKCα binding assay **[I]**. In $^{45}Ca^{2+}$ uptake measurements verapamil hydrochloride (Orion Pharmaceuticals, Finland) and nimodipine (RBI Research Biochemicals International, USA) were used as model compounds with known calcium antagonistic activity **[II, III]**. Ketoprofen (ICN Biomedicals, USA) and paracetamol (Orion Pharmaceuticals, Finland) were used as standard compounds in studying the permeability of the test compounds in the Caco-2 cell monolayer **[V]**. [$^{14}$C]-mannitol (Amersham Pharmacia Biotech, UK) was used as a paracellular transport marker in the Caco-2 studies **[V]**.

The accuracy of the time-resolved fluorometric immunoassay (TR-FIA) developed **[IV]** was evaluated by determining the susceptibility of *Chlamydia pneumoniae* to a set of antimicrobial agents. Erythromycin, rifampicin (Sigma-Aldrich Co., USA), doxycycline, ofloxacin, minocycline, and ciprofloxacin (ICN Biomedicals, USA) were chosen for the study to represent agents with potent antichlamydial activity, whereas streptomycin (Sigma-Aldrich Co., USA) and penicillin G (Fluka Chemie, Switzerland) served as inactive model compounds. To investigate the possibility of detecting compound cytotoxicity simultaneously with the TR-FIA, camptothecin (ICN Biochemicals, USA) and Triton X-100 (Sigma-Aldrich Co., USA) were used as model compounds exhibiting cytotoxic effects.

### 4.1.2. Natural compounds and derivatives

A set of natural compounds and their derivatives were used as the library for the compounds needed for the studies **[I-III, V]**. The compounds were used for screening against two PKC assays **[I]** and for validating the miniaturised $^{45}Ca^{2+}$ uptake assay **[II, III].** Permeability experiments in the Caco-2 cell monolayer and membrane affinity in phospholipid vesicles were conducted for a selected group of flavonoids and alkyl gallates **[V]**. The classification and sources of all the natural compounds and derivatives used in the present study are listed in Table 4. For the experiments, the compounds were dissolved in DMSO to prepare stock solutions, which were diluted to concentrations required for the assays **[I-III, V]** with the appropriate assay buffer.

**Table 4.** Classification and sources of the natural compounds and derivatives used in studies **I-III** and **V**.

| Compound | Used in | Source |
|---|---|---|
| *Catechins and derivatives* | | |
| (+)-Catechin | **I** | Sigma-Aldrich Co., USA |
| (+/-)-Catechin | **I** | Sigma-Aldrich Co., USA |
| (-)-Epicatechin | **I** | Sigma-Aldrich Co., USA |
| (-)-Epicatechin gallate | **I** | Extrasynthese S.A., France |
| (-)-Epigallocatechin | **I** | Extrasynthese S.A., France |
| (-)-Epigallocatechin gallate | **I** | Extrasynthese S.A., France |
| Procyanidin B1 | **I** | Extrasynthese S.A., France |
| Procyanidin B2 | **I** | Extrasynthese S.A., France |
| *Coumarins* | | |
| Daphnetin | **I** | Extrasynthese S.A., France |
| Imperatorin | **III** | Extrasynthese S.A., France |
| 6-Methylcoumarin | **I** | Extrasynthese S.A., France |
| Scopoletin | **I, II** | Sigma-Aldrich Co., USA |
| *Flavanones* | | |
| Hesperidin | **I** | Extrasynthese S.A., France |
| Naringenin | **I, II, V** | Sigma-Aldrich Co., USA |
| Naringin | **I, V** | Extrasynthese S.A., France |
| *Flavones* | | |
| Acacetin | **I, II** | Carl Roth GmbH & Co., Germany |
| Apigenin | **I, II** | Fluka Chemie, Switzerland |
| Baicalin | **I** | Extrasynthese S.A., France |
| Flavone | **I, II, V** | Carl Roth GmbH & Co., Germany |
| Luteolin | **I, II, V** | Extrasynthese S.A., France |
| Vitexin | **I** | Extrasynthese S.A., France |

**Table 4.** Classification and sources of the natural compounds and derivatives used in studies **I-III** and **V**. (cont.)

| Compound | Used in | Source |
|---|---|---|
| *Flavonols* | | |
| Isorhamnetin | **I** | Extrasynthese S.A., France |
| Kaempferol | **I, II** | Extrasynthese S.A., France |
| Morin | **I, II, V** | Carl Roth GmbH & Co., Germany |
| Myricetin | **I, II** | Extrasynthese S.A., France |
| Myricitrin | **I** | Extrasynthese S.A., France |
| Quercetagetin | **I** | Carl Roth GmbH & Co., Germany |
| Quercetin | **I, II, V** | Merck & Co., Germany |
| Quercitrin | **I** | Carl Roth GmbH & Co., Germany |
| Rhamnetin | **I** | Extrasynthese S.A., France |
| Rutin | **III** | Merck & Co., Germany |
| *Isoflavones* | | |
| Daidzein | **I** | Extrasynthese S.A., France |
| Genistein | **I, II** | Extrasynthese S.A., France |
| *Phenolic acids and derivatives* | | |
| Benzoic acid | **I** | Merck & Co., Germany |
| Caffeic acid | **I** | Sigma-Aldrich Co., USA |
| Ferulic acid | **I** | Extrasynthese S.A., France |
| Gallic acid | **I** | Sigma-Aldrich Co., USA |
| Protocatechuic acid | **I** | Carl Roth GmbH & Co., Germany |
| Rosmarinic acid | **I** | Extrasynthese S.A., France |
| Sinapic acid | **I** | Fluka Chemie, Switzerland |
| Syringic acid | **I** | Sigma-Aldrich Co., USA |
| Dodecyl gallate | **I, II** | Fluka Chemie, Switzerland |
| Methyl gallate | **I, II, V** | Fluka Chemie, Switzerland |
| Octyl gallate | **III** | Extrasynthese S.A., France |
| | **I, II, V** | Fluka Chemie, Switzerland |
| Propyl gallate | **I, II, V** | Sigma-Aldrich Co., USA |

## 4.2. Plant material and extraction

Extracts of *Peucedanum palustre* and *Pinus sylvestris* were used to demonstrate the applicability of the combination of micro-fractionation and $^{45}Ca^{2+}$ uptake assay in detecting calcium antagonistic components of natural products **[III]**. Roots of milk parsley *Peucedanum palustre* (L.) Moench belonging to the family Apiaceae (Umbelliferae) were collected in Kirkkonummi, Finland during June and July 2000, and identified by Docent K. Fagerstedt of the Division of Plant Physiology, University of Helsinki, Finland. Voucher specimens are deposited at the Faculty of Pharmacy,

University of Helsinki, Finland (KN 1-3 and KN 6). Air-dried and powdered *Peucedanum palustre* root material (1 g) was extracted with 3 × 10 ml of methanol-water (80:20, v/v) (sonication 10 min, centrifugation 1500 × g, 10min). The combined supernatants were evaporated (water bath 35°C) to a volume of about 2 ml and lyophilised to dryness. The dried extract was dissolved in a mixture of methanol and DMSO (1:1, v/v) to yield a final concentration of 20 mg/ml.

Ground cortex of Scots pine *Pinus sylvestris* (L.) belonging to the family Pinaceae was obtained in August 2000 from the company Ravintorengas (Finland). The ground cortex was produced according to the company's quality requirements. The material was extracted with acetone-water (70:30, v/v) for 48 hours. The extract was lyophilised and the raw extract was further purified using liquid-liquid extraction with n-hexane, chloroform and n-butanol, respectively. The sample was finally collected and lyophilised to be used in this study.

## 4.3. Assays for protein kinase C activity [I]

The potential inhibitory effects of natural compounds and their derivatives on PKC were investigated by exploiting kinase activity and [$^{3}$H]-phorbol ester binding assays. Particular emphasis was placed on demonstrating the suitability of these assays for screening using quality parameters and determinations of repeatability and reproducibility.

### 4.3.1. Kinase activity assay

The inhibitory effects of the model compounds on the kinase activity of rat brain PKC were measured by the incorporation of [$^{32}$P] from [$\gamma$-$^{32}$P]-adenosine triphosphate (ATP) into a PKC-specific substrate. The method has been described in detail by Chakravarthy et al. (1991). Aliquots of partially purified rat brain homogenate were preincubated with model compounds or staurosporine for 10 min at 30°C in a 96-well plate. The assay was initiated by addition of reaction mixture to each well, and after incubation for 5 min at 30°C, the reaction was stopped by pipetting an aliquot of the reaction mixture into a Millipore Multiscreen®-HV 96-well filtration plate (Millipore Corp., USA) fitted with phosphocellulose membrane filters. After washing, the

remaining radioactivity was measured by liquid scintillation counting (Microbeta® Trilux, Perkin-Elmer Life and Analytical Sciences/Wallac Oy, Finland).

### 4.3.2. [$^3$H]-phorbol ester binding assay

The ability of the test compounds to compete with [$^3$H]-phorbol ester in binding to the regulatory domain of PKCα (a PKC isoform) was determined according to Gopalakrishna et al. (1992). Insect cell line derived from *Spodoptera frugiperda* (Sf9) expressing the human PKCα was used as the source of PKCα. Total lysate from Sf9 cells was preincubated with the model compounds or PMA for 10 min at room temperature in a Millipore Multiscreen®-HV 96-well filtration plate (Millipore Corp., USA). The assay was initiated by addition of reaction mixture, and the plate was incubated for 10 min. Proteins were precipitated with 20% polyethyleneglycol and after 15 min incubation the filters were washed and the plates were dried. Inhibition of [$^3$H]-phorbol ester binding was determined by measuring the sample-associated radioactivity by liquid scintillation counting (Microbeta® Trilux, Perkin-Elmer Life and Analytical Sciences/Wallac Oy, Finland).

## 4.4. Miniaturised $^{45}Ca^{2+}$ uptake assay [II, III]

Depolarisation-induced $^{45}Ca^{2+}$ uptake assay in clonal rat pituitary cell line $GH_4C_1$, previously used on the Petri dish scale to measure the calcium channel modulating effects of compounds such as flavonoids (Törnquist and Tashjian 1989, Summanen et al. 2001), was miniaturised into a 96-well plate format. Cell seeding density (range $2 \times 10^4$ to $1 \times 10^5$ cells/well) and culture time (from 1 to 7 days) were optimised by following cell proliferation using protein determination according to Bradford (1976). After optimisation, cells were seeded at a density of $8 \times 10^4$ cells/well, cultured in the plates for 3 days before the experiments, refed once and assayed following the procedure of Törnquist and Tashjian (1989) as shown in Fig. 4. Cell-associated radioactivity was measured using a liquid scintillation counter (Microbeta® Trilux, Perkin-Elmer Life and Analytical Sciences/Wallac Oy, Finland). The validity of the miniaturised assay was checked by measuring the inhibitory effects of known calcium antagonists (verapamil and nimodipine) and a set of natural compounds comprising mainly flavonoids. The suitability of the assay for automation was

evaluated by conducting a series of experiments on a Biomek FX workstation (Beckman Coulter, Inc., USA).

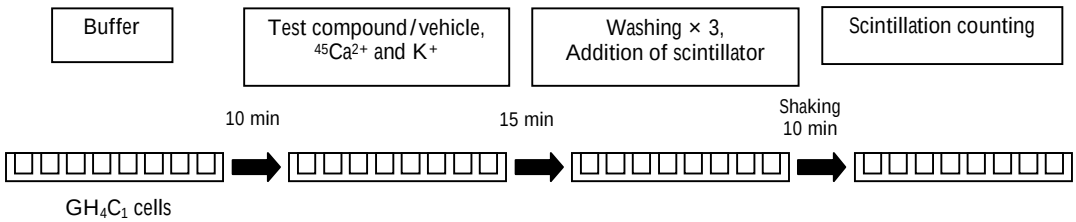


**Figure 4.** The protocol for performing the $^{45}Ca^{2+}$ uptake assay in clonal rat pituitary cell line $GH_4C_1$ in 96-well format.

### 4.4.1. Coupling of miniaturised $^{45}Ca^{2+}$ uptake assay with HPLC micro-fractionation [III]

The miniaturised $^{45}Ca^{2+}$ uptake assay was coupled to HPLC micro-fractionation of a reference solution (containing imperatorin, octyl gallate, rutin and verapamil) and crude plant extracts. The HPLC separations were carried out using a Discovery® HS C18 column (25 cm x 4.6 mm i.d., 5 µm, Supelco, USA) on a Perkin-Elmer Series 200 LC pump and autosampler (USA) with a 200 µl sample loop, Perkin-Elmer LC 235 C Diode Array Detector (USA) at wavelengths of 255 nm and 320 nm and a PE Nelson 600 series link (USA). The mobile phase consisted of methanol and 0.1% (v/v) aqueous formic acid at a flow rate of 1 ml/min. A linear gradient (from 5 to 95% methanol - 0.1% (v/v) aqueous formic acid) of 15 and 20 min followed by a 10-min isocratic period were used for the reference solution and for the plant extracts, respectively. Micro-fractionation of the samples was performed with an automated fraction collector (Gilson FC 204, USA) connected to the HPLC system. The fractions were collected in 96-well microplates with a well volume of 330 µl. Fractionation was performed as a function of time, with a collection time of 0.3 minutes per well. Fractionated samples were lyophilised for 24 hours, dissolved in assay buffer, and the calcium antagonistic activity of the fractions was measured with the $^{45}Ca^{2+}$ uptake assay.

## 4.5. Assays for susceptibility testing of *C. pneumoniae* [IV]

### 4.5.1. Cell culture and infection of cells with *C. pneumoniae*

Cells originating from the human respiratory tract (HL) were used as host cells for the propagation of *C. pneumoniae* Kajaani 7 and for the susceptibility experiments. The cells were seeded in 96-well plates in the TR-FIA experiments and in 24-well plates in the immunofluorescence (IF) microscopy and real-time PCR experiments. Confluent cell monolayers were inoculated with *C. pneumoniae* elementary bodies (EB) by centrifugation (550 × g, 60 min) and after 72 h incubation the infection rate of the cultures was determined by TR-FIA, IF microscopy or real-time PCR.

### 4.5.2. Time-resolved fluorometric immunoassay (TR-FIA)

To increase the throughput in susceptibility testing of chlamydia, a time-resolved fluorometric immunoassay was developed and validated. In the TR-FIA chlamydial inclusions in 96-well plates are stained with a genus-specific murine monoclonal antibody (Anti-Chlamydia genus no. 11-113, clone 5, Argene S.A., France) labelled with isothiocyanate-activated europium (Eu)-DTTA chelate at Wallac Oy (Finland). The *C. pneumoniae* inoculum concentration and labelling conditions (incl. label concentration, incubation time and temperature) were optimised using quality parameters to evaluate the assay performance. In the optimised TR-FIA the inclusions were labelled with 100 µl of the TR-FIA antibody (conc. 100 ng/ml), incubated for 30 min at 37°C and washed six times with 300 µl of Wallac DELFIA® Wash Solution (Perkin-Elmer Life and Analytical Sciences/Wallac Oy, Finland) using a Biohit BW50 plate-washer (Biohit Plc, Finland). An aliquot of 100 µl of DELFIA® Enhancement Solution (Perkin-Elmer Life and Analytical Sciences/Wallac Oy) was added and the plate shaken for 5 min at low mode on a DELFIA® Plateshake (Perkin-Elmer Life and Analytical Sciences/Wallac Oy). Chlamydia-associated TRF signals were measured using a protocol optimised for europium in a Victor$^2$ multilabel counter (excitation/emission 340/615 nm, counting delay and window 400 µs, counting cycle 1000 µs) (Perkin-Elmer Life and Analytical Sciences/Wallac Oy). A schematic illustration of the TR-FIA protocol is presented in Fig. 5. The reliability of the assay was further confirmed by determining the susceptibilities of *C. pneumoniae* reference strain Kajaani 7 to a set of antimicrobials by the TR-FIA and by two reference methods,

IF microscopy and real-time PCR. The possibility of determining the cytotoxic effects of test compounds simultaneously with the TR-FIA was assessed by incorporating a protein dye SRB (Molecular Probes Inc., USA) into the TR-FIA labelling solution at a concentration of 0.8% (w/v). Absorbance at 550 nm was used to calculate loss of cells from treated wells as compared with untreated controls to evaluate the possible cytotoxicity of the samples (see also chapter 4.7).

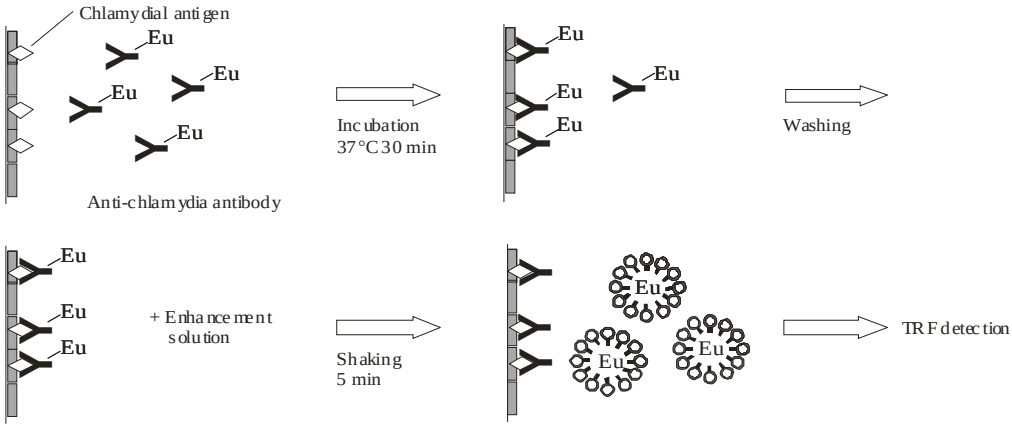


**Figure 5.** Schematic illustration of the TR-FIA protocol.

### 4.5.3. Immunofluorescence (IF) microscopy

For IF staining the HL cells were infected on cover slips and the chlamydial inclusions labelled with genus-specific fluorescein isothiocyanate-conjugated monoclonal antibody (PathFinder® Chlamydia Culture Confirmation System, Bio-Rad S.A., USA). The chlamydial inclusions shown as green fluorescence were examined using a fluorescence microscope with an excitation filter bandwidth of 510-560 nm and a 590 nm barrier filter (Nikon ECLIPSE TE300 inverted microscope with TE-FM epi-fluorescence attachment, Japan). Inclusions in four randomly chosen fields (magnification × 200) were counted from duplicate samples in each minimum inhibitory concentration (MIC) determination.

### 4.5.4. Real-time PCR

DNA from infected cells was extracted using a Qiagen DNA mini-kit (Qiagen Inc., USA). Real-time PCR was used to measure the *C. pneumoniae* DNA present in the samples using the FastStart DNA Master SYBR Green I kit (Roche Biochemicals, Germany), VD-4 primers (Tib molbiol, Germany) and the conditions described by Tondella et al. (2002). Amplification and detection of the PCR product were performed with a standard procedure of a LightCycler instrument (Roche Biochemicals) using the following thermal cycling conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The PCR product was detected as an increase in fluorescence of the SYBR Green dye after the PCR extension phase as a result of binding to newly synthesised double-stranded DNA. The amount of fluorescence is proportional to the amount of DNA, and by measuring the fluorescence after each cycle the increase in DNA concentration in the samples can be monitored in real time.

## 4.6. Assays for biomembrane interactions [V]

### 4.6.1. Permeability studies in Caco-2 cell monolayers

The human colon adenocarcinoma cell line Caco-2 [American Type Culture Collection (ATCC) # HTB-37] was used to evaluate the transport of flavonoids and alkyl gallates across a cell monolayer. For the experiments, the cells were cultured on polycarbonate membranes for 21-27 days. During differentiation, Caco-2 cells form a monolayer with well-distinguishable apical and basolateral membranes, and can be used as an *in vitro* model of the intestinal barrier. A schematic illustration of the principal drug transport mechanisms across the intestinal epithelium is presented in Fig. 6. In this study, the apical-to-basolateral transport of a set of flavonoids and alkyl gallates was investigated with Caco-2 cell monolayers. During the course of this study, the assay format was miniaturised from 12-well to 24-well plates. After washing with physiological Hanks' balanced salt solution (HBSS), the test compounds were added in buffer to the apical compartment and samples from the apical and basolateral compartments were collected at several time points to measure the transport. The samples were analysed by reversed phase HPLC using octadecyl silica stationary phases. For samples containing flavonoids and alkyl gallates a gradient elution (from 5 to 95% methanol - 0.1% aqueous formic acid) of 10 min followed by a 10-min isocratic section

with diode array detection at a wavelength of 255 nm was used. Ketoprofen and paracetamol samples were analysed by isocratic elution with mobile phases consisting of acetonitrile - 0.03% phosphoric acid (50:50, v/v) or water-methanol-acetic acid (65:35:0.4, v/v), respectively, and detected with an absorbance detector at a wavelength of 254 nm (ketoprofen) or 245 nm (paracetamol).

Based on the HPLC results, the apparent permeability coefficients, $P_{app}$ (cm/s) across the cell monolayers were calculated, if transport was detected, according to the equation $P_{app} = (\Delta Q/\Delta t)/(60 \times A \times C_o)$, where $\Delta Q/\Delta t$ is the flux of the compound across the monolayer, 60 the conversion from minutes to seconds, $A$ ($cm^2$) the surface area of the insert, and $C_o$ the initial compound concentration in the apical compartment. Monolayer integrity was assessed during the experiments by transepithelial electrical resistance (TEER) measurements and by [$^{14}$C]-mannitol permeation across the cell monolayer.

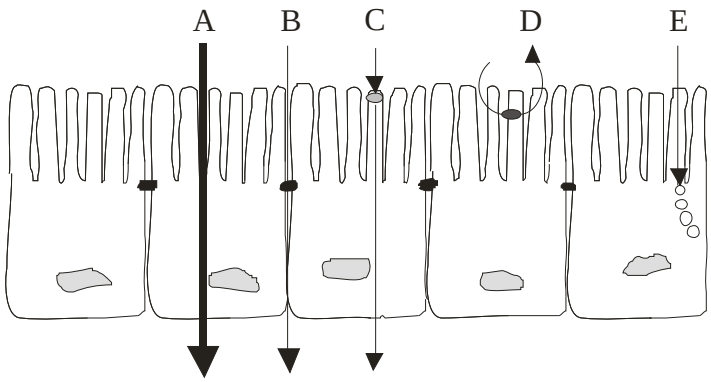


**Figure 6.** Schematic presentation of intestinal epithelium and principal drug transport mechanisms: (A) passive transcellular diffusion, (B) passive paracellular diffusion, (C) active transport, (D) efflux, and (E) endocytosis (adapted from Artursson et al. 1996 and Lindmark 1997).

### 4.6.2. Membrane affinity experiments

The work was carried out at the Department of Biochemistry and Pharmacy (Åbo Akademi University, Turku, Finland). Membrane affinity experiments using bilayer model membranes comprising 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) as the bilayer-forming

phospholipids were used to study association of the model compounds with the membranes. Methods based on fluorescence quenching were employed to determine the partition coefficients (given as $K_d$). The method was originally based on procedures previously described by Verkman (1980) and van Dijk et al. (2000). $K_d$ values were obtained by gradual addition of model compounds into vesicles with the fluorescent probe diphenylhexatriene (used for flavones and flavonols) or *p*-terphenyl (used for naringenin, ketoprofen and paracetamol).

## 4.7. Cytotoxicity assays [II, IV, V]

In this study, the cytotoxicity of the model compounds was determined prior to the experiments to eliminate the possibility of false positives caused by compound cytotoxicity. The assays used were: 1) LDH assay described by Korzeniewski and Callewaert (1983) **[II]**, 2) SRB assay used for the assessment of drug-induced cytotoxicity through measurement of the protein content of cells (Skehan et al. 1990) **[IV]**, and 3) MTT assay measuring the metabolic activity of the cells according to Mosmann (1983) **[V]**.

## 4.8. Data analysis

After screening at a single concentration, dose-response analyses were performed for compounds showing potent activity **[I, II]**. The concentrations yielding 50% inhibition ($IC_{50}$) were determined by fitting the data into four parameter logistic curves using SigmaPlot 2002 for Windows Version 8.0 software (SPSS Inc., USA). The susceptibility results in **[IV]** were reported as MIC values, i.e. the lowest concentration of the antimicrobial agent at which the chlamydia infection was inhibited.

The repeatability and reproducibility of the assays [**I, II, IV]** were evaluated by determining plate-to-plate and day-to-day variations in the results obtained for positive controls. The suitability for screening was further assessed by calculating quality parameters S/N and S/B ratios and Z' factors according to the equations described in Bollini et al. (2002) and in Zhang et al. (1999) (see chapter 2.7). The optimisation and validation data were used to set case-specific thresholds for the parameters to be used in future experiments for the evaluation of data quality **[I, II]**.

# 5. RESULTS

The main results obtained during the course of this study are summarised in the following section.

## 5.1. Screening of protein kinase C inhibition and binding [I]

A group of natural compounds and their derivatives (43 compounds in total) were screened in this study to evaluate their inhibitory effects on PKC with two enzymatic assays based on 1) measuring the kinase activity of partially purified rat brain extract and 2) measuring the binding of [$^{3}$H]-phorbol ester to recombinant human PKCα.

***Inhibition of protein kinase C activity.*** The inhibition of kinase activity was studied by measuring the ability of PKC to incorporate [$^{32}$P] from [γ-$^{32}$P]-ATP into a PKC-specific substrate in the presence of the test compound. Nine compounds out of the 43 examined exhibited significant inhibition of PKC activity. The most potent inhibitors were the catechins (-)-epigallocatechin gallate and (-)-epicatechin gallate which had $IC_{50}$ values of 4.8 and 5.9 µM, respectively. Five flavonols (myricetin, quercetin, rhamnetin, isorhamnetin and kaempferol) and one flavone (luteolin) also showed potent inhibition, the $IC_{50}$ values ranging from 7.8 to 33.7 µM (Table 5).

**Table 5.** Inhibition of protein kinase C activity by the most potent inhibitors given as concentrations resulting in 50% inhibition ($IC_{50}$) in the kinase activity of partially purified rat brain extract.

| **Compound** | **$IC_{50}$ (µM)** |
|---|---|
| Dodecyl gallate | 8.9 |
| (-)-Epicatechin gallate | 5.9 |
| (-)-Epigallocatechin gallate | 4.8 |
| Isorhamnetin | 26.5 |
| Kaempferol | 33.7 |
| Luteolin | 15.5 |
| Myricetin | 7.8 |
| Quercetin | 13.5 |
| Rhamnetin | 14.4 |
| Staurosporine (positive control) | 0.003 |

The gallic acid derivative dodecyl gallate was also found to be a good inhibitor of PKC ($IC_{50}$ of 8.9 µM), while the derivatives with shorter side chains, e.g. methyl and propyl gallate, caused only modest or negligible inhibition. Insignificant effects were noted for coumarins, flavanones, isoflavones and simple phenolic acids **[I, Fig. 1]**.

***Inhibition of phorbol ester binding.*** Phorbol ester binds to the regulatory domain of PKC causing activation. Most of the diversity between different PKC isoforms is in the regulatory domain (Mellor and Parker 1998) and the binding to this site could be an indication of isoform selectivity. None of the compounds used in this study competed effectively with the binding of [$^3$H]-phorbol ester to the regulatory domain of PKCα **[I, Table 1]**. The most potent were (-)-epigallocatechin gallate, rhamnetin, (-)-epicatechin gallate and dodecyl gallate, which produced inhibitions of 59%, 49%, 45%, and 40%, respectively, at a concentration of 150 µM. These results would indicate that the PKC inhibition seen in the kinase activity assay was mainly due to binding to the catalytic domain of PKC.

***Assay quality and performance.*** The second specific aim of the study **[I]** was to evaluate quality parameters for the two assays, i.e. how well they are suited for screening. Reproducibility was assessed on the basis of results relating to positive controls, which were staurosporine for the kinase activity assay and PMA for the [$^3$H]-phorbol ester binding assay. Plate-to-plate and day-to-day variations in the kinase activity were 3.3% ($n$ = 3 in duplicate) and 10.1% ($n$ = 10), and for the [$^3$H]-phorbol ester binding assay 0.5% ($n$ = 3 in duplicate) and 3.7% ($n$ = 4), respectively. Assay quality parameters S/B, S/N and Z' factor were 16.1, 11.3, and 0.62 ($n$ = 14) for the kinase activity assay and 47.4, 15.9 and 0.73 ($n$ = 10) for the [$^3$H]-phorbol ester binding assay, respectively. These figures indicate that both assays are sensitive, reproducible, and able reliably to detect PKC inhibition. Z' factor values above 0.5 show the suitability of the assays for screening.

## 5.2. Assay development for screening of calcium channel blocking activity [II, III]

The clonal rat pituitary cell line $GH_4C_1$ possesses a high concentration of L- type voltage-operated calcium channels (VOCCs) similar to those found in cardiovascular tissue (Tan and Tashjian 1984,

Wagner et al. 1993). The VOCCs mediate a variety of biological processes in the cardiovascular, central nervous system (CNS) and metabolic areas, making these channels a highly important, physiologically relevant target for screening of inhibitors (Denyer et al. 1998). The $GH_4C_1$ cell line has been extensively used on the Petri dish scale for studying the calcium antagonistic properties of compounds and natural products (Rauha et al. 1999, Haansuu et al. 1999, Haansuu et al. 2001, Summanen et al. 2001). However, the method previously employed is very laborious and has a low throughput.

***Assay miniaturisation.*** The assay for studying calcium uptake in $GH_4C_1$ cells was miniaturised into 96-well plate format and the assay conditions relating to cell culture were optimised **[II]**. Cell proliferation in the 96-well plate was assessed by using the protein content of the wells as a measure of cellular growth. Wells were seeded with starting cell densities ranging from $2\times 10^4$ to $1 \times 10^5$ cells/well, and cellular growth was followed for 7 days by protein determination. Cellular growth reached the same plateau level of approx. $3 \times 10^5$ cells/well after 3 days when $8 \times 10^4$ and $1 \times 10^5$ cells were seeded per well, and after 4 days with a starting density of $6 \times 10^4$ cells/well **[II, Fig. 1; erroneously $10^5$]**. As a result, the incubation time prior to experiments could be reduced from 7 to 3 days. The seeding density $8 \times 10^4$ was found to be the optimum. The edge-wells were omitted from the experiment due to variable cellular behaviour in these wells seen as increased data variation.

***Assay validation and performance.*** The reliability of the assay in measuring calcium channel activity was evaluated using the calcium antagonists verapamil and nimodipine together with a set of natural compounds and their derivatives (15 compounds in total) as model compounds. $IC_{50}$ values for nimodipine and verapamil obtained with the miniaturised assay were 0.007 and 3.4 µM, respectively. Four compounds inhibiting the $^{45}Ca^{2+}$ flux were identified from amongst the natural compounds and derivatives: octyl gallate, naringenin, flavone and genistein. The most potent of these compounds was octyl gallate, with an $IC_{50}$ of 34 µM **[II, Table 2]**.

The majority of the compounds were observed to be calcium channel activators, the most effective being myricetin (99% increase in $^{45}Ca^{2+}$ entry, Fig. 7) **[II, Table 3]**. Myricetin was also able to increase the $^{45}Ca^{2+}$ flux by 42% when the experiment was performed without $K^+$ depolarisation, whereas the more moderate activators, i.e. apigenin, kaempferol and dodecyl gallate, had no effect on the $^{45}Ca^{2+}$ flux without $K^+$ stimulation. The assay can easily be modified to study the mechanism

of activation in more detail, e.g. by the addition of H-89, a cyclic 3',5'-adenosine monophosphate (cAMP)-dependent protein kinase A (PKA)-inhibitor, to influence the upmodulation of VOCCs by cAMP-dependent PKA. Of the activators, myricetin and dodecyl gallate were able to induce an increase in the $^{45}Ca^{2+}$ uptake under H-89 suppression, which suggests that they act through a cAMP-independent pathway.

In summary, the results obtained agreed well with our previous data (Härmälä et al. 1992, Haansuu et al. 2001, Summanen et al. 2001) demonstrating the ability of the miniaturised assay to yield reliable information on the calcium channel modulating effects of compounds.

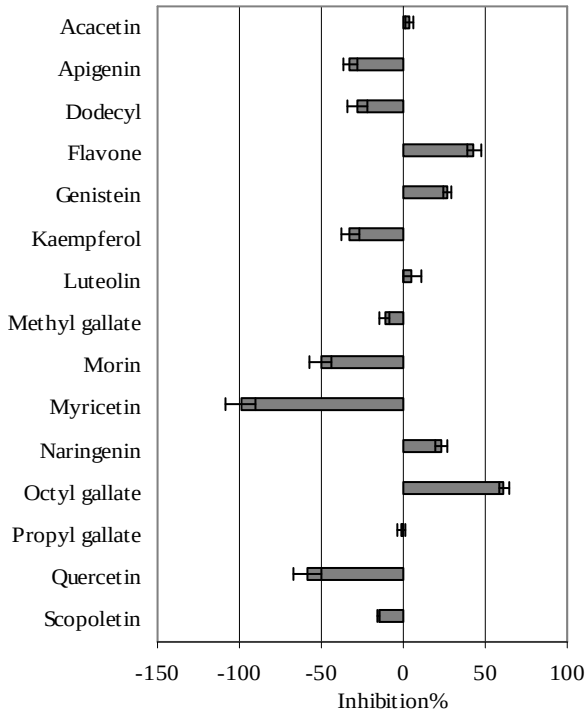


**Figure 7.** The effects of the model compounds (at a concentration of 50 µM) on $^{45}Ca^{2+}$ uptake in $GH_4C_1$ cells determined with the 96-well plate assay (the bars show the mean ± S.E.M. from two to four separate experiments, each with six replicates).

The suitability for screening was evaluated on the basis of quality parameters and data repeatability and reproducibility. The S/B and S/N ratios averaged 3.9 and 10.3, respectively, and the Z' factor 0.59 ($n$ = 9) in the series of experiments performed with the model compounds. Verapamil was used as a positive control during the course of this study, and was included at a concentration of 40 µM (yielding an average inhibition of 88%) on every assay plate and employed in assessing the repeatability and reproducibility of the results. The plate-to-plate and day-to-day variability in the results obtained for verapamil were 4.2 ($n$ = 3 in triplicate) and 5.0% ($n$ = 5), respectively.

***Assay automation.*** The suitability of the cell-based $^{45}Ca^{2+}$ uptake assay for automation was evaluated by running a series of experiments on a Biomek FX workstation. Markedly lower values for the quality parameters and higher variability of results were observed in the automated compared with the manual experiments (Fig. 8). The S/B, S/N, and Z' factors were 2.6, 5.3, and 0.22 ($n$ = 5), respectively, for the automated version of the assay. However, this did not have a significant effect on the $IC_{50}$ values obtained for the calcium channel inhibitors. From the automated experiments the $IC_{50}$ values for verapamil and nimodipine were 3.7 and 0.010 µM, respectively, whereas the manual experiments yielded values of 3.4 and 0.007 µM.

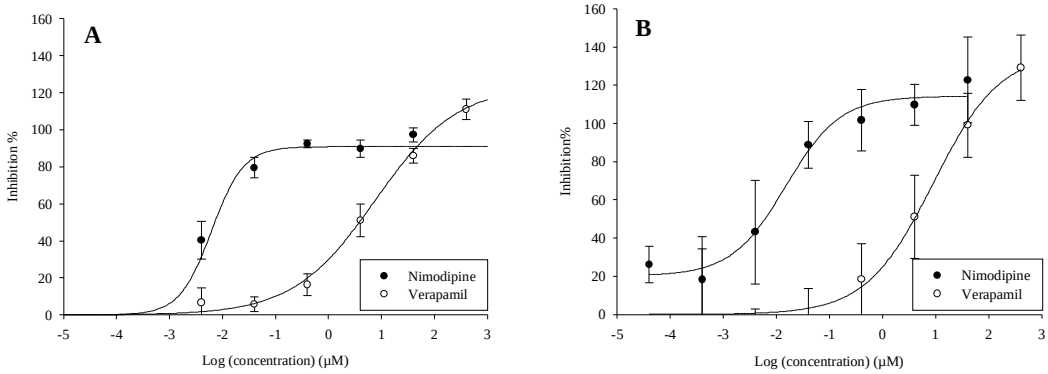


**Figure 8.** Dose-response curves for verapamil and nimodipine determined by (A) manual and (B) automated $^{45}Ca^{2+}$ uptake assay in $GH_4C_1$ cells. Data points represent the mean ± standard deviation of six replicates.

***Coupling of miniaturised $^{45}Ca^{2+}$ uptake assay to HPLC micro-fractionation.*** The automated, miniaturised $^{45}Ca^{2+}$ uptake assay in $GH_4C_1$ cells was successfully coupled to HPLC micro-fractionation of plant extracts [**III]**. With a reference solution containing verapamil, octyl gallate, imperatorin and rutin, the accuracy of the integrated assay in detecting calcium channel antagonists and the effects of micro-fractionation and lyophilisation were assessed. The theoretical final concentration of the compounds was calculated to be 16 µg/ml in the $^{45}Ca^{2+}$ uptake assay. These concentrations yielded inhibitions of 100, 93, and 79 for verapamil, imperatorin, octyl gallate, respectively, when the effects of these compounds on the $^{45}Ca^{2+}$ uptake were measured without a prior HPLC micro-fractionation step. No effect was observed with rutin. For the micro-fractionated reference solution, the following inhibitory effects were detected: verapamil 34-79% (fractionated into 5 wells), imperatorin 6-42% (fractionated into 3 wells), octyl gallate 11-49% (fractionated into 2 wells), and rutin showed no effect **[III, Fig. 1]**. Clearly, these results indicate that the HPLC micro-fractionation did not cause any significant loss of activity in the model compounds, although direct comparison of results between unfractionated and fractionated samples was not possible due to the fractionation of one compound into several wells on the plate. Based on the experiments made with the reference solution, the threshold for considering a substance active was set at 30%.

To further show the suitability of the assay for diverse types of samples, primary detection of calcium antagonistic components from plant extracts was performed. This would also be beneficial when working with other complex mixtures such as synthetic mixtures and fermentation broths. A root extract of *Peucedanum palustre* causing a total inhibition of $^{45}Ca^{2+}$ flux, and *Pinus sylvestris* extract showing no activity were chosen as model extracts for the experiments. The HPLC micro-fractionation of the extracts was performed, the fractions were lyophilised, dissolved in assay buffer and subjected to the $^{45}Ca^{2+}$ uptake assay. HPLC chromatograms were combined with activity profiles enabling us to detect the active components direct from the chromatogram **[III, Fig. 2]**. The calcium channel blocking activity of *P. palustre* root extract could be linked to components identified by HPLC-UV, LC-MS and retention time as isoimperatorin and columbianadin. Columbianadin has previously been reported to be the most active calcium channel inhibiting compound in the root extract of *P. palustre* (Vuorela 1988). As anticipated, no significantly inhibitory components were detected from the *P. sylvestris* extract (inhibitions under 18%) **[III, Fig. 3]**.

## 5.3. Assay development for screening of antichlamydial activity [IV]

A novel time-resolved fluorometric immunoassay (TR-FIA) was created to increase the sample throughput in the screening of antichlamydial activity. The assay is based on a europium-conjugated, genus-specific antibody, which allows a multilabel counter to be used for measuring the presence of chlamydial inclusions in the samples. By employing time-resolved fluorometry as the detection technology, the interfering autofluorescence from the host cells could be eliminated through temporal resolution.

***Assay development and optimisation.*** The assay was miniaturised from the previous 24-well plate format to a 96-well plate format in which the seeding of $6 \times 10^4$ HL cells/well was shown to result in confluent monolayers when grown overnight prior to infection. On the basis of experiments with a series of *C. pneumoniae* inoculum concentrations ranging from $0.26 \times 10^3$ to $26 \times 10^6$ inclusion-forming units (IFU)/ml, the cells were infected in the optimised TR-FIA protocol with an inoculum of $4 \times 10^5$ IFU/ml. This yielded excellent values for the quality parameters (S/B = 5.07, S/N = 9.27, Z' = 0.66) and a reliably high infection rate as visually determined by IF staining experiments. The TR-FIA was able to detect an inoculum of $2.6 \times 10^3$ IFU/ml, corresponding to 90 IFU/well, but the concentration should be greater than $26 \times 10^3$ IFU/ml before reliable difference between infected and non-infected cells can be obtained if evaluated by the signal and the quality parameters **[IV, Fig. 1, Table 2]**. With the aim of improving the assay performance, optimal labelling conditions as regards label concentration, incubation time and temperature were determined by calculating the quality parameters from the data obtained for each set of conditions **[IV, Fig. 1, Table 1]**. From the results, a concentration of 100 ng/ml and an incubation of 30 min at 37°C were selected. With these conditions, the quality parameters were: S/B 6.84, S/N 6.69 and Z' 0.52.

***Assay quality and performance.*** The TR-FIA showed excellent repeatability and reproducibility for a cell-based assay. Using erythromycin (7.8 ng/ml) as a positive control, plate-to-plate variation was 8.9% ($n$ = 3) and day-to-day variation 9.0% ($n$ = 3). Variation in the infection rate within one plate was investigated by infecting all 96 wells of one plate. When all the wells were included, the variation in the measured signal corresponding to the infection rate was 18%, but this was reduced to 12% when the edge-wells were excluded (signals measured from individual wells are presented in Fig. 9). Apparently, the 3-day incubation during the infection caused significant evaporation in

the edge-wells and led to divergent results for the infection rate. For this reason the edge-wells were not used in the subsequent experiments.

Data on the quality parameters was collected during the validation experiments. The average S/B and S/N ratios were 6.48 and 6.85, respectively, and the Z' factor 0.39 ($n$ = 19). Although not ideal, these values can be regarded as good for a cell-based assay in which there is a second source of biological variation, i.e. chlamydia. The assay is thus suitable for screening but the quality parameters should be carefully examined after each experiment to detect any problems with the experimental outcome, i.e. the success of infection.

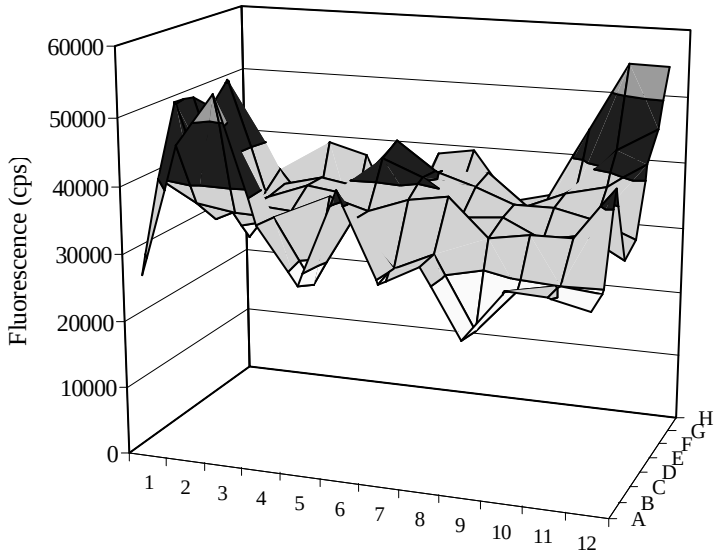


**Figure 9.** Within-plate variability of the maximum signal in the TR-FIA. The 3D surface graph has been constructed from signals measured from individual wells of one 96-well plate. The signals have been categorised and the levels are presented with different colours in the graph, dark colours representing high and light colours low signal levels. The numbers and letters on the X and Z axes represent the column and row titles on the plate, respectively.

***Assay validation.*** The susceptibility (measured as MICs) of *C. pneumoniae* Kajaani 7 to a set of antimicrobial agents was determined using the TR-FIA, IF microscopy and real-time PCR to show the reliability of the TR-FIA. In summary, MICs determined using the three methods agreed well, with TR-FIA and IF microscopy yielding almost identical results **[IV, Table 3]**. Real-time PCR seemed to give slightly lower MIC values than the other two methods, but the differences were generally only one dilution lower and thus insignificant. The three most potent agents in inhibiting chlamydial infection were rifampicin, erythromycin and doxycycline, with MIC values of 0.008 (0.004 according to PCR), 0.016 and 0.063 µg/ml, respectively. The quinolones ciprofloxacin and ofloxacin inhibited infection, with MIC values of 0.5-1.0 µg/ml, and a MIC of 0.063 µg/ml (0.031 µg/ml for PCR) was obtained for minocycline. Penicillin G and streptomycin were found to be inactive. The former showed no inhibition at concentrations up to 72 µg/ml according to TR-FIA and IF staining, whereas the MIC was 18 µg/ml according to PCR. No activity was observed with streptomycin at concentrations of 288 µg/ml (72 µg/ml for PCR).

***Simultaneous cytotoxicity assessment.*** Cytotoxicity of the test compounds can result in false positives, and should therefore be evaluated in cell-based screens. The best way to perform the cytotoxicity assessment would be to combine it with the actual bioassay. In the TR-FIA the addition of SRB to the TR-FIA antibody solution enabled the concomitant cytotoxicity assay. SRB is a dye which can be used to evaluate the loss of cells caused by test compound treatment. Despite some interference between the TR-FIA antibody and SRB, the assay was shown to be reliable when dual labelling with the SRB was performed (S/B = 3.18, S/N = 5.55, Z' = 0.35). The cytotoxicity of camptothecin and Triton X-100 causing overestimation of chlamydial susceptibility was clearly detectable with the dual labelling assay **[IV, Fig. 2]**.

## 5.4. Comparison of membrane interactions in Caco-2 cells and in phospholipid vesicles [V]

The biomembrane interactions of a set of flavonoids and alkyl gallates (11 in total) were examined using transport studies on Caco-2 cells and membrane affinity experiments in phospholipid vesicles. The degree of hydroxylation, molecular configuration, and length of the side chain were found to markedly influence the membrane partition and permeability characteristics of these

compounds. Flavonoids with no or up to three free hydroxyl groups were transported across the Caco-2 cell monolayer, i.e. flavone and naringenin with $P_{app}$ values of $380 \times 10^{-6}$ and $29.4 \times 10^{-6}$ cm/s **[V, Table 1]**, respectively, whereas flavonoids with several hydroxyl groups, e.g. quercetin and luteolin, were rapidly taken up by the cells but not transported to the basolateral compartment **[V, Fig. 2].** The apical concentration of catechin isomers (+)-catechin and (-)-epicatechin was not reduced during the 90-min incubation and were thus unable to penetrate the cell membrane. Polyhydroxylated flavonoids exhibited the highest affinity for POPC vesicles, the partition coefficients ($K_d$) being 7.1 and 7.5 µM for luteolin and quercetin, respectively. On the other hand, the membrane affinity of flavone was weak ($K_d$ = 115 µM). In conclusion, high membrane affinity seemed to result in poor transepithelial transport of the compound. The degree of hydroxylation and molecular configuration were seen to be critical factors in membrane affinity and the transcellular transport of flavonoids.

# 6. DISCUSSION

## 6.1. Relevance of the targets

The assays employed in this study were targeted at the evaluation of protein kinase C (PKC) activity **[I]**, $^{45}Ca^{2+}$ uptake via voltage-gated calcium channels (VOCCs) **[II, III]**, the susceptibility of *Chlamydia pneumoniae* **[IV]** and biomembrane interactions **[V]**.

The family of PKC isozymes are important signal transducers in a number of cellular responses including cell proliferation and tumorigenesis and have been connected with various pathological disorders including cancer, heart disease and diabetes (Newton 1997). PKCα, one of the PKC isoforms, has been reported to suppress apoptosis in several cell lines (Dean et al. 1996, Connolly and Rose 1997, Gutcher et al. 2003). It has also been predicted that PKCs will be major drug targets in the twenty-first century (Cohen 2002). Selective PKC inhibitors have been described for the treatment of diabetic retinopathy (Engel et al. 2000, Cohen 2002).

Voltage-operated calcium channels are found in cardiac, neuronal and skeletal tissues and have a crucial role in the regulation of excitation-contraction coupling in smooth muscle (Tsien and Tsien 1990, Denyer et al. 1998). Calcium channel inhibitors are used in treating angina, arrhythmia, hypertension, migraine, and atherosclerosis (Denyer et al. 1998). Indeed, 15% of the 100 top-selling drugs worldwide are ion channel modulators (England 1999). The clonal rat pituitary cell line, $GH_4C_1$, used in this study **[II, III]**, possesses a high concentration of L-type VOCCs (Tan and Tashjian 1984). Depolarisation-induced $^{45}Ca^{2+}$ uptake through voltage-gated calcium channels has been well characterised in $GH_4C_1$ cells (Tan and Tashjian 1984) and treatment with known calcium channel blockers causes a high inhibition of the $Ca^{2+}$ flux, making this cell line an excellent model for studying the calcium channel modulating effects of compounds.

*C. pneumoniae* is an obligate intracellular bacterium causing acute infections such as pharyngitis, bronchitis and pneumonia as well as chronic infections associated with various diseases, including atherosclerosis (Saikku et al. 1988, Grayston et al. 1995), asthma (Hahn et al. 1991, Hahn et al. 1996) and CNS symptoms (Koskiniemi et al. 1996). No specific treatment for *C. pneumoniae* infections exists and chronic infections have been found to be extremely difficult to diagnose and

treat with current antimicrobial agents. Screening for new potential antichlamydial agents has therefore unquestionable importance, but has been hampered by the lack of a high-throughput assay for *C. pneumoniae* susceptibility testing. Development of the novel TR-FIA **[IV]**, the first higher throughput method for screening antichlamydial activity, greatly benefits the methodology and speeds up the screening process.

Biomembrane interactions and cellular membrane permeability are of pivotal importance to the activity of the compounds employed in cell-based assays as well as for their bioavailability (Seydel 2002). Oral absorption characteristics are usually estimated *in vitro* using differentiated monolayer cultures of human colon adenocarcinoma cell line Caco-2 to mimic the monolayer of intestinal epithelial cells lining the gastrointestinal tract (Artursson and Karlsson 1991, Artursson et al. 1996). Due to the laboriousness of this method, several other techniques have been investigated as possible alternatives, including parallel artificial membrane permeation assay (PAMPA), surface plasmon resonance biosensor technique, the use of immobilised artificial membrane (IAM) columns, and partitioning into immobilised liposomes (Seydel 2002, Di and Kerns 2003). In the present study, the Caco-2 model and a biochemical assay measuring the partitioning into phospholipid vesicles were used to study a group of flavonoids and alkyl gallates to compare the functionality of these methods in predicting the biomembrane interactions of these compounds **[V]**.

## 6.2. Biochemical versus cell-based assays

An assay for bioactivity screening can be based on examining either isolated targets such as purified enzymes or proteins or targets in a cellular environment. The two PKC assays utilised in this study were based on a mixture of enzymes extracted from rat brain and on a recombinant PKCα expressed in baculovirus-infected insect cells **[I]**. The membrane affinity experiments were conducted using POPC vesicles **[V]**. In the three cell-based assays, cell lines $GH_4C_1$ **[II, III]**, HL **[IV]** and Caco-2 **[V]** were employed.

The benefits of the biochemical assays are quite clearly seen in this study: they show excellent reproducibility and performance in terms of quality parameters and are significantly faster to perform (e.g. no cell culture time is needed prior to experiments). On the other hand, the knowledge obtained is quite limited; potent inhibitors could be recognised but this does not yet

give any indications as to whether these compounds will ultimately function in a cellular environment. In fact, the two most prominent PKC inhibitors identified **[I]** were catechin derivatives, and for similar compounds both cellular uptake and permeability in Caco-2 experiments were shown to be very slow **[V],** which should raise some concerns over their functionality.

Direct information on the ability of compounds to affect cellular targets such as VOCCs **[II, III]** or on their cytotoxicity **[II, IV, V]** can be achieved using cell-based assays for screening. More complicated assay systems are usually needed, which places additional requirements on assay design, development and optimisation. During experiments, cells may change their biological properties and response due to any experimental variability, such as temperature, pH, media, serum concentration or cell density, so that particularly careful planning of procedures is a necessity for cell-based assays (Boguslavsky 2004b). As shown by the optimisation of the cell culture procedure for the $^{45}Ca^{2+}$ uptake in $GH_4C_1$ cells **[II]**, the determination of optimal cell seeding density and growth phase of the cells can have a significant effect on the cell culture needs for screening. The high within-plate variation and edge-effects in signal noted with the two 96-well format cellular assays developed in this study **[II, IV]** is a result of variations in cell growth across the assay plate during long incubations, probably caused by temperature variations or media evaporation. Compound cytotoxicity can lead to false positives, and should thus always be examined in cellular assays. The best approach would be the simultaneous determination of cytotoxicity and the actual biological activity to be evaluated, such as the coupling of a cell proliferation assay to antibody labelling **[IV]**.

Cell-based assays are also irreplaceable in situations where the specific target for pharmacological intervention has not been recognised, which is the case in infections caused by chlamydia **[IV]**. On the other hand, one of the disadvantages of using cell-based screens is that it is hard to determine the exact mechanism of compound activity (Oldenburg et al. 2001).

The ability of compounds to interact with biomembranes, i.e. their cellular permeability, is of great importance during the screening process. The $P_{app}$ values from the Caco-2 experiments and the partition coefficients from the membrane affinity experiments yielded similar information on the biomembrane interactions of flavonoids **[V]**, i.e. strong membrane affinity was generally accompanied by poor apical to basolateral transport in Caco-2 cells. Thus, at least with these model

compounds, the membrane affinity experiments would have been a good predictor of a compound's permeability characteristics and a useful tool to replace the tedious Caco-2 model. Even though it is possible to perform the Caco-2 experiments in miniaturised formats such as 24- and 96-well density (unpublished results), the throughput of the assay, including HPLC analysis of the samples, is still much more laborious. As it is significantly faster to perform, the biochemical membrane affinity assay could be considered as an alternative for eliminating compounds with undesirable characteristics from large compound pools.

## 6.3. Advances in assay development, throughput and automation

In seeking to improve the throughput of cell-based assays used in bioactivity screening, this study led to several significant achievements. The miniaturisation of $^{45}Ca^{2+}$ uptake in $GH_4C_1$ cells from Petri dish scale to 96-well plate format, and optimisation of the cell culture practice, resulted in a decrease in culture time from 7 to 3 days and an increase in the number of data points obtained from one assay from 30 to 60. Furthermore, 80% less cells and 90% less reagents are needed compared to the Petri dish assay, which means substantial savings, including in the use of $^{45}Ca^{2+}$ **[II, Table 1]**. Developing a 96-well plate assay (TR-FIA) for screening antichlamydial activity meant valuable improvements to the traditional 24-well plate assay based on IF staining (Table 6). The miniaturisation from 24-well to 96-well format resulted in significant savings in cell amounts and use of *C. pneumoniae* inoculum, while at the same time increasing the number of data points obtainable from one experiment 2.5-fold.

**Table 6.** Summary on the benefits achieved through assay miniaturisation and automation for screening of antichlamydial activity.

| **Assay conditions** | **TR-FIA** | **IF staining** |
|---|---|---|
| Assay volume (µl) | 200 | 1000 |
| Cell amount | 60,000 | 400,000 |
| Use of *C. pneumoniae* inoculum (IFU/well) | 14,000 | 80,000 |
| Data points per assay | 60 | 24 |
| Analysis time | 3 min | hours |
| Number of steps exploiting automation* | 8 | - |

*here the automation is considered as the use of multichannel and multidose pipettors and stand-alone devices in sample handling

The objectivity of the TR-FIA is also a great benefit compared to IF staining. In the TR-FIA, several steps in the assay protocol are automated and enable the handling of multiple samples simultaneously, making the protocol far less tedious than IF staining and PCR. Owing to the use of a multilabel counter, the results of one plate in the TR-FIA can be obtained significantly faster than with conventional IF staining (Table 6).

Automation of liquid handling operations and other steps in the assay protocol is one of the key requirements in HTS assays (Oldenburg et al. 2001). In the present study, the assay for $^{45}Ca^{2+}$ uptake in $GH_4C_1$ cells was shown to be suitable for automated operation by a liquid handling device **[II]**. In the manual version, liquid handling was based on multichannel pipetting, which already enabled the simultaneous handling of parallel samples. The automation was taken further by introducing the assay to an automated environment in which the pipetting steps were performed with a 96-channel pipetting head. The experiments performed on a Biomek FX workstation demonstrated that when a manually operated assay is taken to an automated platform, the protocol needs to be carefully re-evaluated and optimised **[II]**. Clearly, liquid handling plays a critical role in the performance of automated cell-based assays.

The coupling of the automated $^{45}Ca^{2+}$ uptake assay to analytical HPLC micro-fractionation greatly facilitates the classical process leading from a plant to a pharmacologically active, pure constituent **[III]**. Using HPLC micro-fractionation, components of crude extracts can be divided into fractions, collected into micro-well plates and subjected to diverse bioassays to examine properties such as enzyme inhibition or antimicrobial effects (Hamburger 2003, Wennberg et al. 2004). With this approach the observed bioactivity can be connected to the biologically relevant component by identifying it from the chromatogram on the basis of the bioactivity profile obtained, avoiding the isolation of inactive compounds. One notable benefit in the automation suitability of the integrated assay, in addition to the reduction in work-load, is the fact that the fractionation is made into 96-well plates, which are directly compatible with automated liquid handling, and thus the samples can be taken direct to the assay from the plate with no need for prior sample transfer.

In summary, the development of a screening assay for the evaluation of biological activity is a process where several decisions need to be considered. Factors connected with the different steps of the process are presented in Fig. 10.

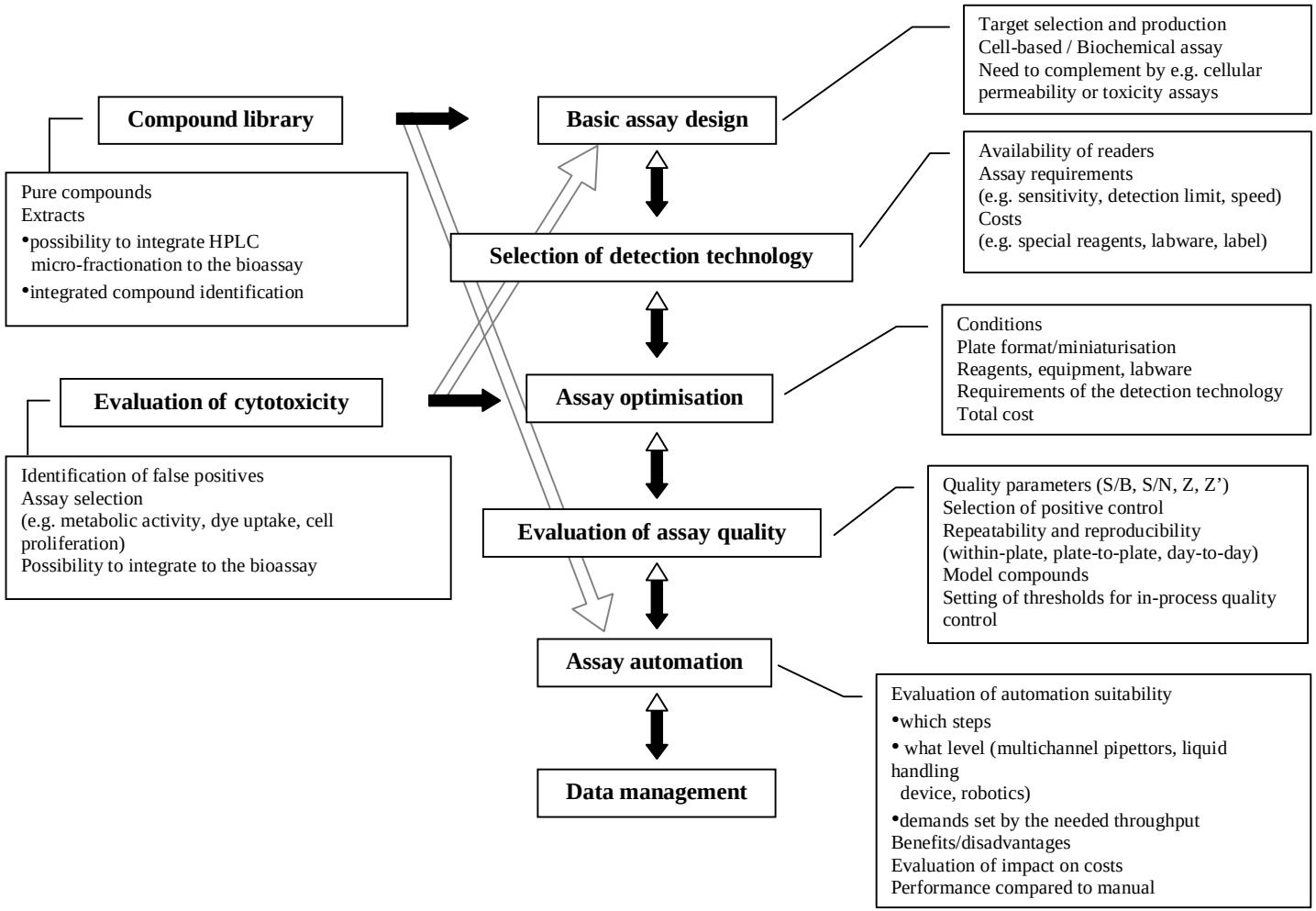


**Figure 10.** Scheme on the factors involved in assay development for preclinical screening process in academic settings.

## 6.4. Quality parameters in assay development and validation

Special requirements are set on assays used for bioactivity screening, particularly on those used in HTS. Most of all, the assays need to be robust, reproducible and suitable for automation (Sittampalam et al. 1997a, Zhang et al. 1999). A simple way to evaluate these features is to use the quality parameters S/B, S/N and Z' factor. When isolated targets were employed, as in PKC assays **[I]**, values of S/B 47.4, S/N 15.9, and Z' 0.73 for the [$^{3}$H]-phorbol ester binding assay were obtained (Table 7). The day-to-day variation of the assay was 3.7%, indicating good reproducibility. In the case of cell-based assays, the biological variation has an impact and lower values are typical (e.g. the cell-based $^{45}Ca^{2+}$ uptake assay yielding S/B 3.9, S/N 10.3, and Z' 0.59, and day-to-day variation of 5.0%, Table 7). As shown by the results, the response obtainable in cellular experiments can have limitations (e.g. the S/B ratio can be weaker), and in fact even when similar cell lines and reporter gene constructs are utilised, the activities measured can be significantly different (Zhang et al. 1999). Moreover, in the case of a highly complex assay system such as chlamydial infection in HL cells in the TR-FIA **[IV]**, the values are further affected (S/B 6.5, S/N 6.9, Z' 0.39, and day-to-day variation 9.0%).

**Table 7.** Summary of the values obtained for the quality parameters **[I, II, IV].**

| Assay | S/B | S/N | Z' | Day-to-day variation (%) |
|---|---|---|---|---|
| Kinase activity | 16.1 | 11.3 | 0.62 | 10.1 |
| [$^{3}$H]-Phorbol ester binding | 47.4 | 15.9 | 0.73 | 3.7 |
| $^{45}Ca^{2+}$ uptake | 3.9 | 10.3 | 0.59 | 5.0 |
| TR-FIA | 6.5 | 6.9 | 0.39 | 9.0 |

The assay quality also depend greatly on the detection technique employed in the assay. The increase in signal obtained with a good label and a suitable detection technique can significantly improve the difference between the signal of interest and the interfering background, the result being better assay performance in terms of the quality parameters. There are several alternative technologies, each of which has certain advantages. One of these is TRF, in which lanthanide chelates are used to enable separation of autofluorescence relating to cells from the actual signal (Soini and Kojola 1983, Hemmilä et al. 1984). This approach was found to be a powerful tool in developing the TR-FIA for screening of antichlamydial activity **[IV]**. Discrimination between

infected and non-infected cells was not possible using a fluorescein isothiocyanate-labelled antibody due to the weakness of the signal and the interfering host cells, whereas a good S/B ratio was obtained by exploiting a europium-labelled antibody. Through detailed optimisation of labelling conditions, the quality of the assay was further enhanced. The choice of detection technique and conditions can thus have a major impact on the quality of the assay.

# 7. CONCLUSIONS

Assay development for bioactivity screening has strong impacts on the final outcome of the whole drug discovery process. The present study focused on several aspects related to assay design and quality with the aim of setting up a panel of assays for performing bioactivity screening in an academic setting.

When developing a bioassay, careful consideration must be given to several issues that may affect the quality of the assay. Depending on the target, the complexity of its function and other factors, one of the biggest decisions is the choice between biochemical and cell-based screens. This study utilised both enzyme- and cell-based assays, each of which has certain benefits. The protein kinase C assays clearly showed that one important advantage of assays exploiting isolated targets over cell-based assays is their better reproducibility. Secondly, in cell-based assays, the cell culture infrastructure will certainly limit the sample throughput rate. On the other hand, cellular formats yield biologically more relevant information on efficacy, e.g. direct effects on $Ca^{2+}$ channels in $GH_4C_1$ cells, and simultaneous observation of a compound's cytotoxicity. The biomembrane experiments with flavonoids and alkyl gallates showed that in certain situations there are potential alternative ways to examine the biological function. The relevance of information from more laborious and time-consuming cell-based assays needs to be thoroughly considered on a case-by-case basis.

Another central decision in assay design is the choice of detection technique. Advances in the development of detection methods have significantly boosted the use of more complex cell-based assays at early stages in bioactivity screening programmes. Radiometric labelling, although being used less and less because of safety and health concerns, has the ability to yield high S/B ratios and is more suitable for certain assay types than fluorescence-based methods, e.g. for the PKC and $^{45}Ca^{2+}$ uptake assays in this study. Then again, the diversity of sophisticated fluorometric methods offers multiple opportunities to overcome many problems associated with cell-based assays. The unique properties of time-resolved fluorometry enabled us to design a miniaturised cell-based immunoassay, TR-FIA, for screening antichlamydial activity. Using this approach the assay was miniaturised from 24- to 96-well format, thus enabling the simultaneous handling of multiple

samples, automated washing after staining, and, most importantly, plate reader-based detection as well as simultaneous cytotoxicity testing.

Detailed optimisation of assays can lead to a significant improvement in assay quality such as stronger signals in immunoassays, and it is therefore important to find the optimum assay conditions and cell culture procedures. During assay development, the repeatability and reproducibility need to be ascertained by determining within-plate, plate-to-plate and day-to-day variations. The quality parameters S/B, S/N, and Z' are a valuable set of tools in bioactivity screening. In assay development, different assay conditions can easily be compared in relation to the quality of the data obtained. Also, during the screening phase, the reliability of the data can be checked by monitoring the values of quality parameters from individual experiments.

In terms of saving time and money, the miniaturisation of assays by changing to high-density plate formats facilitates the screening of large compound libraries. However, this often necessitates a higher degree of assay automation when the manual handling of plates and reagent additions become impossible. In the case of cell-based assays, moving to automated assay format requires additional optimisation of assay design, as we clearly evidenced during automation of the $^{45}Ca^{2+}$ uptake measurement. In our studies, the automated assay was further coupled with HPLC micro-fractionation, helping us to identify calcium antagonist components in a root extract of *Peucedanum palustre* and excluding the preparative chromatographic separation previously needed.

The achievements of this study in terms of assay development, miniaturisation and automation will certainly be useful for evaluating PKC inhibitory, calcium antagonistic and antichlamydial activities as well as the biomembrane interactions of test compounds. Most importantly, the knowledge gained from developing and optimising the bioassays will be beneficial for setting up bioactivity screening projects in academic settings.

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**REFERENCES**


Aalto, J., Eskola, A., Kaihola, L., Mottram, P., Rundt, K., Stark, T., Suontausta, J., Yrjönen, T., Scintillation counting. In: Bioanalytical applications of labelling technologies, Eds. Hemmilä, I., Ståhlberg, T., Mottram, P., Wallac Oy, Turku, (1994) pp. 53-82.

Andreani, A., Cavalli, A., Granaiola, M., Guardigli, M., Leoni, A., Locatelli, A., Morigi, R., Rambaldi, M., Recanatini, M., Roda, A., Synthesis and screening for antiacetylcholinesterase activity of (1-benzyl-4-oxopiperidin-3-ylidene)methylindoles and –pyrroles related to donepezil. J. Med. Chem. (2001) 44, 4011-4014.

Artursson, P., Karlsson, J., Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem. Biophys. Res. Commun. (1991) 175, 880-885.

Artursson, P., Palm, K., Luthman, K., Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv. Drug Deliv. Rev. (1996) 22, 67-84.

Auer, M., Moore, K.J., Meyer-Almes, F.J., Guenther, R., Pope, A.J., Stoeckli, K.A., Fluorescence correlation spectroscopy: lead discovery by miniaturized HTS. Drug Discov. Today (1998) 3, 457-465.

Banerjee, A., Kaul, P., Sharma, R., Banerjee, U.C., A high-throughput amenable colorimetric assay for enantioselective screening of nitrilase-producing microorganisms using pH sensitive indicators. J. Biomol. Screen. (2003) 8, 559-565.

Beske, O.E., Goldbard, S., High-throughput cell analysis using multiplexed array technologies. Drug Discov. Today (2002) 7, S131-S135.

Biazzo-Ashnault, D.E., Park, Y.W., Cummings, R.T., Ding, V., Moller, D.E., Zhang, B.B., Qureshi, S.A., Detection of insulin receptor tyrosine kinase activity using time-resolved fluorescence energy transfer technology. Anal. Biochem. (2001) 291, 155-158.

Bleicher, K.H., Bohm, H.J., Muller, K., Alanine, A.I., Hit and lead generation: beyond high-throughput screening. Nat. Rev. Drug Discov. (2003) 2, 369-378.

Blomberg, K., Granberg, C., Hemmilä, I., Lövgren, T., Europium-labelled target cells in an assay of natural killer activity. I. A novel non-radioactive methods based on time-resolved fluorescence. J. Immunol. Methods (1986) 86, 225-229.

Boguslavsky, J., HTS assay development: is smaller really better? Drug Discov. Dev. (2004a) 7, 37-40.

Boguslavsky, J., HTS assay development: cellular assays and targets. Drug Discov. Dev. (2004b) 7, 35-39.

Bollini, S., Herbst, J.J., Gaughan, G.T., Verdoorn, T.A., Ditta, J., Dubowchik, G.M., Vinitsky, A., High-throughput fluorescence polarization method for identification of FKBP12 ligands. J. Biomol. Screen. (2002) 7, 526-530.

Bosse, R., Illy, C., Elands, J., Chelsky, D., Miniaturizing screening: how low can we go today? Drug Discov. Today: HTS Suppl. (2000) 1, 42-47.

Bouma, G.J., Van der Meer-Prins, P.M.W., Van Bree, F.P.M.J., Van Rood, J.J., Claas, F.H.J., Determination of cytotoxic T-lymphocyte precursor frequencies using europium labelling as nonradioactive alternative to labelling with chromium-51. Hum. Immunol. (1992) 35, 85-92.

Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. (1976) 72, 248-254.

Bronson, D., Hentz, N., Janzen, W.P., Lister, M.D., Menke, K., Wegrzyn, J., Sittampalam, G.S., Basic considerations in designing high-throughput screening assays. In: Handbook of drug screening. Seethala, R. and Fernandes, P.B. Eds. Marcel Dekker Inc., New York, (2001) pp. 5-30.

Brown, J.A., Marala, R.B., Development of a high-throughput screening-amenable assay for human poly(ADP-ribose) polymerase inhibitors. J. Pharmacol. Toxicol. Methods (2002) 47, 137-141.
Brown, D., Superti-Fuga, G., Rediscovering the sweet spot in drug discovery. Drug Discov. Today (2003) 8, 1067-1077.
Burbaum, J.J., Miniaturisation technologies in HTS: how fast, how small, how soon? Drug Discov. Today (1998) 3, 313-322.
Butcher, S.P., Target discovery and validation in the post-genomic era. Neurochem. Res. (2003) 28, 367-371.
Chakravarthy, B.R., Bussey, A., Whitfield, J.F., Sikorska, M., Williams, R.E., Durkin, J.P., The direct measurement of protein kinase C (PKC) activity in isolated membranes using a selective peptide substrate. Anal. Biochem. (1991) 196, 144-150.
Cohen, P., Protein kinases – the major drug targets of the twenty-first century? Nat. Rev. Drug Discov. (2002) 1, 309-315.
Comley, J., Assay interference – a limiting factor in HTS? Drug Discov. World (2003) Summer, 91-98.
Connolly, J.M., Rose, D.P., Expression of the invasive phenotype by MCF-7 human breast cancer cells transfected to overexpression protein kinase C-α or the erbB2 proto-oncogene. Int. J. Oncol. (1997) 10, 71-76.
Cooper, M.A., Biosensor profiling of molecular interactions in pharmacology. Curr. Opin. Pharmacol. (2003) 3, 557-562.
Danz, H., Stoyanova, S., Wippich, P., Brattström, A., Hamburger, M., Identification and isolation of the cyclooxygenase-2 inhibitory principle in *Isatis tinctoria*. Planta Med (2001) 67, 411-416.
Davies, N., Peakman, T., Arlington, S., A new formula for finding drugs. Drug Discov. Today (2004) 9, 197-199.
Dean, N., McKay, R., Miraglia, L., Howard, R., Cooper, S., Giddings, J., Nicklin, P., Meister, L., Ziel, R., Geiger, T., Muller, M., Fabbro, D., Inhibition of growth of human tumor cell lines in nude mice by an antisense oligonucleotide inhibitor of protein kinase C-α expression. Cancer Res. (1996) 56, 3499-3507.
Denyer, J., Worley, J., Cox, B., Allenby, G., Banks, M., HTS approaches to voltage-gated ion channel drug discovery. Drug Discov. Today (1998) 3, 323-332.
Di, L., Kerns, E.H., Profiling drug-like properties in discovery research. Curr. Opin. Chem. Biol. (2003) 7, 402-408.
Dickson, M., Gagnon, J.P., Key factors in the rising cost of new drug discovery and development. Nat. Rev. Drug Discov. (2004) 3, 417-429.
van Dijk, C., Driessen, A.J.M., Recourt, K., The uncoupling efficiency and affinity of flavonoids for vesicles. Biochem. Pharmacol. (2000) 60, 1593-1600.
DiMasi, J.A., Hansen, R.W., Grabowski, H.G., The price of innovation: new estimates of drug development costs. J. Health Econ. (2003) 22, 151-185.
Dove, A., Screening for content – the evolution of high throughput. Nat. Biotechnol. (2003) 21, 859-864.
Drews, J., Genomic sciences and the medicine of tomorrow. Nat. Biotechnol. (1996) 14, 1516-1518.
Drews, J., Drug discovery: A historical perspective. Science (2000) 287, 1960-1964.
Drews, J., Strategic trends in the drug industry. Drug Discov. Today (2003) 8, 411-420.
van Duin, M., Woolson, H., Mallinson, D., Black, D., Genomics in target and drug discovery. Biochem. Soc. Trans. (2003) 31, 429-432.

Duncan, R.R., Bergmann, A., Cousin, M.A., Apps, D.K., Shipston, M.J., Multi-dimensional time-correlated single photon counting (TCSPC) fluorescence lifetime imaging microscopy (FLIM) to detect FRET in cells. J. Microsc. (2004) 215, 1-12.

Earnshaw, D.L., Moore, K.J., Greenwood, C.J., Djaballah, H., Jurewicz, A.J., Murray, K.J., Pope, A.J., Time-resolved fluorescence energy transfer DNA helicase assays for high throughput screening. J. Biomol. Screen. (1999) 4, 239-248.

Eggeling, C., Brand, L., Ullmann, D., Jäger, S., Highly sensitive fluorescence detection technology currently available for HTS. Drug Discov. Today (2003) 8, 632-641.

Ehrenberger, M., Rigler, R., Rotational brownian motion and fluorescence intensity fluctuations. Chem. Phys. (1974) 4, 390-401.

Eigen, M., Rigler, R., Sorting single molecules: Application to diagnostic and evolutionary biotechnology. Proc. Natl. Acad. Sci. USA (1994) 91, 5740-5747.

Elands, J., The evolution of laboratory automation. In: Handbook of drug screening. Seethala, R. and Fernandes, P.B. Eds. Marcel Dekker Inc., New York, (2001) pp. 477-492.

Eldridge, G.R., Vervoort, H.C., Lee,C.M., Cremin, P.A., Williams, C.T., Hart, S.M., Goering, M.G., O'Neil-Johnson, M., Zeng, L., High-throughput method for the production and analysis of large natural product libraries for drug discovery. Anal. Chem. (2002) 74, 3963-3971.

Emptage, N.J., Fluorescent imaging in living systems. Curr. Opin. Pharmacol. (2001) 1, 521-525.

Engel, G.L., Farid, N.A., Faul, M.M., Richardson, L.A., Winneroski, L.L., Salt from selection and characterization of LY333531 mesylate monohydrate. Int. J. Pharm. (2000) 198, 239-247.

England, P.J., Discovering ion-channel modulators – making the electrophysiologist's life more interesting. Drug Discov. Today (1999) 4, 391-392.

Entzeroth, M., Emerging trends in high-throughput screening. Curr. Opin. Pharmacol. (2003) 3, 522-529.

Epps, D.E., Mitchell, M.A., Petzold, G.L., VanDrie, J.H., Poorman, R.A., A fluorescence resonance energy transfer method for measuring the binding of inhibitors to stromelysin. Anal. Biochem. (1999) 275, 141-147.

Falconer, M., Smith, F., Surah-Narwal, S., Congrave, G., Liu, Z., Hayter, P., Ciaramella, G., Keighley, W., Haddock, P., Waldron, G., Sewing, A., High-throughput screening for ion channel modulators. J. Biomol. Screen. (2002) 7, 460-465.

Frank, R.G., New estimates of drug development process. J. Health Econ. (2003) 22, 325-330.

Frantz, S., Smith, A., New drug approvals for 2002. Nat. Rev. Drug Discov. (2003) 2, 95-96.

González, J.E., Negulescu, P.A., Intracellular detection assays for high-throughput screening. Curr. Opin. Biotechnol. (1998) 9, 624-631.

Gopalakrishna, R., Chen, Z.H., Gundimenda, U., Wilson, J.C., Anderson, W.B., Rapid filtration assays for protein kinase C activity and phorbol ester binding using multiwell plates with fitted filtration discs. Anal. Biochem. (1992) 206, 24-35.

Grayston, J.T., Kuo, C.C., Coulson, A.S., Campbell, L.A., Lawrence, R.D., Lee, M.J., Strandness, E.D., Wang, S.P., *Chlamydia pneumoniae* (TWAR) in atherosclerosis of the carotid artery. Circulation (1995) 92, 3397-3400.

Gribbon, P., Sewing, A., Fluorescence readouts in HTS: no gain without pain? Drug Discov. Today (2003) 8, 1035-1043.

Grover, G.S., Turner, B.A., Parker, C.N., Meier, J., Lala, D.S., Lee, P.H., Multiplexing nuclear receptors for agonist identification in a cell-based reporter gene high-throughput screen. J. Biomol. Screen. (2003) 8, 239-246.

Gutcher, I., Webb, P.R., Anderson, N.G., The isoform-specific regulation of apoptosis by protein kinase C. Cell Mol. Life Sci. (2003) 60, 1061-1070.

Haansuu, P., Vuorela, P. and Haahtela, K., Detection of antimicrobial and $^{45}Ca^{2+}$-transport blocking activity in *Frankia* culture broth extracts. Pharm. Pharmacol. Lett. (1999) 9, 1-4.

Haansuu, P., Klika, K., Söderholm, P., Ovcharenko, V., Pihlaja, K., Haahtela, K. and Vuorela, P., Isolation and biological activity of frankiamide. J. Ind. Microbiol. Biot. (2001) 27, 62-66.

Hahn, D.L., Dodge, R.W., Golubjatnikov, R., Association of *Chlamydia pneumoniae* (strain TWAR) infection with wheezing, asthamatic bronchitis, and adult-onset asthma. JAMA (1991) 266, 225-230.

Hahn, D.L., Anttila, T., Saikku, P., Association of *Chlamydia pneumoniae* IgA antibodies with recently symptomatic asthma. Epidemiol. Infect. (1996) 117, 513-517.

Hamburger, M., Tracking bioactivity in plant exctracts – new concepts and approaches. In: Society for Medicinal Plant Research 50 Years 1953-2003, A jubilee edition. Sprecher, E. Ed. Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, (2003) pp. 109-122.

Hardy, L.W., Peet, N.P., The multiple orthogonal tools approach to define molecular causation in the validation of druggable targets. Drug Discov. Today (2004) 9, 117-125.

Harris, S., Transgenic knockouts as part of high-throughput, evidence-based target selection and validation strategies. Drug Discov. Today (2001) 6, 628-636.

Harvey, A., Strategies for discovering drugs from previously unexplored natural products. Drug Discov. Today (2000) 5, 294-300.

Haupts, U., Rüdiger, M., Ashman, S., Turconi, S., Bingham, R., Wharton, C., Hutchinson, J., Carey, C., Moore, K.J., Pope, A.J., Single-molecule detection technologies in miniaturized high-throughput screening: fluorescence intensity distribution analysis. J. Biomol. Screen. (2003) 8, 19-33.

Hemmilä, I., Dakubu, S., Mukkala, V.M., Siitari, H., Lövgren, T., Europium as a label in time-resolved immunofluorometric assays. Anal. Biochem. (1984) 137**,** 335-343.

Hemmilä, I., Choice of the label concept. In: Bioanalytical applications of labelling technologies, Eds. Hemmilä, I., Ståhlberg, T., Mottram, P., Wallac Oy, Turku, (1994) pp. 13-24.

Hemmilä, I., Harju, R., Time-resolved fluorometry. In: Bioanalytical applications of labelling technologies, Eds. Hemmilä, I., Ståhlberg, T., Mottram, P., Wallac Oy, Turku, (1994) pp. 83-119.

Hemmilä, I.A., Hurskainen, P., Novel detection strategies for drug discovery. Drug Discov. Today (2002) 7, S150-S156.

Hertzberg, R.P., Pope, A.J., High-throughput screening: new technology for the 21st century. Curr. Opin. Chem. Biol. (2000) 4, 445-451.

Hopkins, A.L., Groom, C.R., The druggable genome. Nat. Rev. Drug Disc. (2002) 1, 727-730.

Hostettmann, K., Wolfender, J.L., Terreaux, C., Modern screening techniques for plant extracts. Pharm. Biol. (2001) 39 (Suppl.), 18-32.

Hovius,R., Vallotton, P., Wohland, T., Vogel, H., Fluorescence techniques: shedding light on ligand-receptor interactions. Trends Pharmacol. Sci. (2000) 21, 266-273.

Huss, U., Ringbom, T., Perera, P., Bohlin, L., Vasänge, M., Screening of ubiquitous plant constituents for COX-2 inhibition with a scintillation proximity based assay. J. Nat. Prod. (2002) 65, 1517-1521.

Härmälä, P., Vuorela, H., Hiltunen, R., Nyiredy, S., Sticher, O., Törnqvist, K. and Kaltia, S., Strategy for the isolation and identification of coumarins with calcium antagonistic properties from the roots of *Angelica archangelica*. Phytochem. Anal. (1992) 3, 42-48.

Jablonski, A., Efficiency of anti-Stokes fluorescence dyes. Nature (1933) 131, 839-840.

Johnston, P.A., Johnston, P.A., Cellular platforms for HTS: three case studies. Drug Discov. Today (2002) 7, 353-363.

Joyeux, A., Balaguer, P., Germain, P., Boussioux, A.M., Pons, M., Nicolas, J.C., Engineered cell lines as a tool for monitoring biological activity of hormone analogs. Anal. Biochem. (1997) 249, 119-130.

Karvinen, J., Elomaa, A., Mäkinen, M.J., Hakala, H., Mukkala, V.M., Peuralahti, J., Hurskainen, P., Hovinen, J., Hemmilä, I., Caspase multiplexing: simultaneous homogeneous time-resolved quenching assay (TruPoint) for caspases 1, 3, and 6. Anal. Biochem. (2004) 325, 317-325.

Kask, P., Palo, K., Ullmann, D., Gall, K., Fluorescence-intensity distribution analysis and its application in biomolecular detection technology. Proc. Natl. Acad. Sci. USA (1999) 96, 13756-13761.

Kenakin, T., Predicting therapeutic value in the lead optimization phase of drug discovery. Nat. Rev. Drug Discov. (2003) 2, 429-438.

Kim, J., Felts, S., Llauger, L., He, H., Huezo, H., Rosen, N., Chiosis, G., Development of a fluorescence polarization assay for the molecular chaperone Hsp90. J. Biomol. Screen. (2004) 9, 375-397.

Klostermeier, D., Sears, P., Wong, C.H., Millar, D.P., Williamson, J.R., A three-fluorophore FRET assay for high-throughput screening of small-molecule inhibitors of ribosome assembly. Nucleic Acids Res. (2004) 32, 2707-2715.

Koppal, T., Labs have growing interest in cell culture automation. Drug Discov. Dev. (2003) November, 69-71.

Korzeniewski, C., Callewaert, D.M., An enzyme-release assay for natural cytotoxicity. J. Immunol. Methods (1983) 64, 313-320.

Koskiniemi, M., Gencay, M., Salonen, O., Puolakkainen, M., Färkkilä, M., Saikku, P., Vaheri, A., *Chlamydia pneumoniae* associated with central nervous system infections. Eur. Neurol. (1996) 36, 160-163.

Knowles, J., Gromo, G., Target selection in drug discovery. Nat. Rev. Drug Discov. (2003) 2, 63-69.

Kupcho, K., Hsiao, K., Bulleit, B., Goueli, S.A., A homogeneous, nonradioactive high-throughput fluorogenic protein phosphatase assay. J. Biomol. Sreen. (2004) 9, 223-231.

Lakowicz, J.R., Principles of fluorescence spectroscopy. Plenum Press, New York, (1983) pp. 1-496.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. et al., Initial sequencing and analysis of the human genome. Nature (2001) 409, 860-921.

Lehel, C, Daniel-Issakani, S., Brasseur, M., Strulovici, B., A chemiluminescent microtiter plate assay for sensitive detection of protein kinase activity. Anal. Biochem. (1997) 244, 340-346.

Lindmark, T., Drug absorption enhancement in the intestinal epithelium using medium chain fatty acids. Acta Universitatis Upsaliensis, Reprocentralen HSC, Uppsala (1997).

Lipsky, M.S., Sharp, L.K., From idea to market: the drug approval process. J. Am. Board Fam. Pract. (2001) 14, 362-367.

Lövborg, H., Nygren, P., Larsson, R., Multiparametric evaluation of apoptosis: effects of standard cytotoxic agents and the cyanoguanidine CHS 828. Mol. Cancer Ther. (2004) 3, 521-526.

Maiti, S., Haupts, U., Webb, W.W., Fluorescence correlation spectroscopy: Diagnostics for sparse molecules. Proc. Natl. Acad. Sci. USA (1997) 94, 11753-11757.

Mannerström, M., Zorn-Kruppa, M., Diehl, H., Engelke, M., Toimela, T., Mäenpää, H., Huhtala, A., Uusitalo, H., Salminen, L., Pappas, P., Marselos, M., Mäntylä, M., Mäntylä, E., Tähti, H., Evaluation of the cytotoxicity of selected systemic and intravitreally dosed drugs in the cultures of human retinal pigment epithelial cell line and of pig primary retinal pigment epithelial cells. Toxicol. In Vitro (2002) 16, 193-200.

Marron, B.E., Jayawickreme, C.K., Going to the well no more: lawn format assays for ultra-high-throughput screening. Curr. Opin. Chem. Biol. (2003) 7, 395-401.

Martins, T.B., Development of internal controls for the Luminex instrument as part of a multiplexed seven-analyte viral respiratory antibody profile. Clin. Diagn. Lab. Immunol. (2002) 9, 41-45.

McMahon, J.B., Beutler, J.A., O'Keefe, B.R., Goodrum, C.B.B., Myers, M.A., Boyd, M.R., Development of a cyanovirin-N-HIV-1 gp120 binding assay for high throughput screening of natural product extracts by time-resolved fluorescence. J. Biomol. Screen. (2000) 5, 169-176.

Mellor, H., Parker, P.J., The extended protein kinase C superfamily. Biochem. J. (1998) 332, 281-292.

Minta, A., Kao, J.P., Tsien, R.Y., Fluorescent indicator for cytosolic calcium based on rhodamine and fluorescein chromophores. J. Biol. Chem. (1989) 264, 8171-8178.

Moore, K., Rees, S., Cell-based versus isolated target screening: How lucky do you feel? J. Biomol. Screen. (2001) 6, 69-74.

Mossmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods (1983) 65, 55-63.

Naylor, L.H., Reporter gene technology: the future looks bright. Biochem. Pharmacol. (1999) 58, 749-757.

Newman, D.J., Cragg, G.M., Snader, K.M., Natural products as sources of new drugs over the period 1981-2002. J. Nat. Prod. (2003) 66, 1022-1037.

Newton, A.C., Regulation of protein kinase C. Curr. Opin. Cell Biol. (1997) 9, 161-167.

Ng, J.H., Ilag, L.L., Streamlining drug discovery: finding the right drug against the right target to treat the right disease. Drug Discov. Today (2004) 9, 59-60.

Ng, T., Shima, D., Squire, A., Bastiaens, P.I.H., Gschmeissner, S., Humphries, M.J., Parker, P.J., PKCα regulates β1 integrin-dependent cell-motility through association and control of integrin traffic. EMBO J. (1999) 18, 3909-3923.

Nieuwenhuijsen, B.W., Huang, Y., Wang, Y., Ramirez, F., Kalgaonkar, G., Young, K.H., A dual luciferase multiplexed high-throughput screening platform for protein-protein interactions. J. Biomol. Screen. (2003) 8, 676-684.

Nilakantan, R., Nunn, D.S., A fresh look at pharmaceutical screening library design. Drug Discov. Today (2003) 8, 668-672.

Oldenburg, K.R., Kariv, I., Zhang, J., Chung, T.D.Y., Lin, S., Assay miniaturisation, developing technologies and assay formats. In: Handbook of drug screening. Seethala, R. and Fernandes, P.B. Eds. Marcel Dekker Inc., New York, (2001) pp. 525-562.

Pope, A.J., Haupts, U.M., Moore, K.J., Homogeneous fluorescence readouts for miniaturized high-throughput screening: theory and practice. Drug Discov. Today (1999) 4, 350-362.

Rauha, J.-P., Tammela, P., Summanen, J., Vuorela, P., Kähkönen, M., Heinonen, M., Hopia, A., Kujala, T., Pihlaja, K., Törnquist, K. and Vuorela, H., Actions of some plant extracts containing flavonoids and other phenolic compounds on calcium fluxes in clonal rat pituitary $GH_4C_1$ cells, Pharm. Pharmacol. Lett. (1999) 9, 66-69.

Riss, T.L., Moravec, R.A., Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. Assay Drug Dev. Technol. (2004) 2, 51-62.

Roda, A., Guardigli, M., Pasini, P., Mirasoli, M., Bioluminescence and chemiluminescence in drug screening. Anal. Bioanal. Chem. (2003) 377, 826-833.

Rogers, M.V., Light on high-throughput screening: fluorescence-based assay technologies. Drug Discov. Today (1997) 2, 156-160.

Rüdiger, M., Haupts, U., Moore, K.J., Pope, A.J., Single-molecule detection technologies in miniaturized high throughput screening: binding assays for G protein –coupled receptors using fluorescence intensity distribution analysis and fluorescence anisotropy. J. Biomol. Screen. (2001) 6, 29-37.

Saarinen, K., Kivistö, K., Blomberg, K., Punnonen, K., Leino, L., Time-resolved fluorometric assay for leukocyte adhesion using a fluorescence enhancing ligand. J. Immunol. Methods (2000) 236, 19-26.

Saikku, P., Leinonen, M., Mattila, K., Nieminen, M.S., Huttunen, J.K., Valtonen, V., Serological evidence of an association of a novel chalmydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet (1988) 2, 983-986.

Schenk, T., Breel, G.J., Koevoets, P., van den Berg, S., Hogenboom, A.C., Irth, H., Tjaden, U.R., van den Greef, J., Screening of natural products extracts for the presence of phosphodiesterase inhibitors using liquid chromatography coupled online to parallel biochemical detection and chemical characterization. J. Biomol. Screen. (2003) 8, 421-429.

Schmid, E.F., Smith, D.A., Is pharmaceutical R&D just a game of chance or can strategy make a difference? Drug Discov. Today (2004) 9, 18-26.

Seethala, R., Screening platforms. In: Handbook of drug screening. Seethala, R. and Fernandes, P.B. Eds. Marcel Dekker Inc., New York, (2001) pp. 31-67.

Seydel, J.K., Drug-membrane interaction and pharmacokinetics of drugs. In: Drug-membrane interactions. Mannhold, R., Kubinyi, H., Folkers, G., Eds. Wiley-VCH Verlag GmbH, Weinheim, (2002) pp. 141-215.

Simmonds, M.S.J., Novel drugs from botanical sources. Drug Discov. Today (2003) 8, 721-722.

Sittampalam, G.S., Iversen, P.W., Boadt, J.A., Kahl, S.D., Bright, S., Zock, J.M., Janzen, W.P., Lister, M.D., Design of signal windows in high throughput screening assays for drug discovery. J. Biomol. Screen. (1997a) 2, 159-169.

Sittampalam, G.S., Kahl, S.D., Janzen, W.P., High-throughput screening: advances in assay technologies. Curr. Opin. Biotechnol. (1997b) 1, 384-391.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.S., New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. (1990) 82, 1107-1112.

Slater, K., Cytotoxicity tests for high-throughput drug discovery. Curr. Opin. Biotechnol. (2001) 12, 70-74.

Smith, C., Hitting the target. Nature (2003) 422, 341-347.

Soini, E., Kojola, H., Time-resolved fluorometer for lanthanide chelates – A new generation of non-isotopic immunoassays. Clin. Chem. (1983) 29, 65-68.

Stetler-Stevenson, W.G., Aznavoorian, S., Liotta, L.A., Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu. Rev. Cell Biol. (1993) 9, 541-573.

Stohl, W.R., The role of natural products in a modern drug discovery program. Drug Discov. Today (2000) 5, 39-41.

Summanen, J., Vuorela, P., Rauha, J.P., Tammela, P., Marjamäki, K., Pasternack, M., Törnquist, K., Vuorela, H., Effects of simple aromatic compounds and flavonoids on $Ca^{2+}$ fluxes in rat pituitary $GH_4C_1$ cells. Eur. J. Pharmacol. (2001) 414, 125-133.

Sundberg, S.A., High-throughput and ultra-high-throughput screening: solution- and cell-based approaches. Curr. Opin. Biotechnol. (2000) 11, 47-53.

Sundberg, S.A., Chow, A., Nikiforov, T., Wada, H.G., Microchip-based systems for target validation and HTS. Drug Discov. Today (2000) 5, S92-S103.

Tan, K.-N., Tashjian, Jr., A.H., Voltage-dependent calcium channels in pituitary cells in culture: I. Characterization by $^{45}Ca^{2+}$ fluxes. J. Biol. Chem. (1984) 259, 418-426.

Taylor, P.B., Optimizing assays for automated platforms. Modern Drug Discov. (2002) December, 37-39.

Tondella, M.L.C., Talkington, D.F., Holloway, B.P., Dowell, S.F., Cowley, K., Soriano-Gabarro,M., Elkind, M.S., Fields, B.S., Development and evaluation of real-time PCR-based

fluorescence assays for detection of *Chlamydia pneumoniae*. J. Clin. Microbiol. (2002) 40, 575-583.

Trinh, L., Ziegler, R., Watling, D., Snider, Croze, E. Development of an immunofluorometric, high-capacity, cell-based assay for the measurement of human type I and type II interferons. J. Biomol. Screen. (1999) 4, 33-37.

Trinka, R.F., Leichtfried, F.E., Robotics and automation. In: Handbook of drug screening. Seethala, R. and Fernandes, P.B. Eds. Marcel Dekker Inc., New York, (2001) pp. 493-523.

Tsien, R.W., Tsien, R.Y., Calcium channels, stores, and oscillations. Annu. Rev. Cell Biol. (1990) 6, 715-760.

Tulp, M., Bohlin, L., Unconventional natural sources for future drug discovery. Drug Discov. Today (2004) 9, 450-458.

Turconi, S., Bingham, R.P., Haupts, U., Pope, A.J., Developments in fluorescence lifetime-based analysis for ultra-HTS. Drug Discov. Today (2001) 6, S27-S39.

Törnquist, K., Tashjian, A.H., Dual actions of 1,25-dihydroxycholecalciferol on intracellular $Ca^{2+}$ in $GH_4C_1$ cells: Evidence for effects on voltage-operated $Ca^{2+}$ channels and $Na^{+}/Ca^{2+}$ exchange. Endocrinol. (1989) 124, 2765-2776.

Udenfriend, S., Gerber, I., Nelson, N., Scintillation proximity assay: a sensitive and continuous isotopic method for monitoring ligand/receptor and antibody/antigen interactions. Anal. Biochem. (1987) 161, 494-500.

Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A. et al., The sequence of the human genome. Science (2001) 291, 1304-1351.

Verkman, A.S., The quenching of an intramembrane fluorescent probe. A methods to study the binding and permeation of phloretin through bilayers. Biochim. Biophys. Acta (1980) 599, 370-379.

Verkman, A.S., Drug discovery in academia. Am. J. Physiol. Cell Physiol. (2004) 286, C465-C474.

Verpoorte, R., van der Heijden, R., Memelink, J., Engineering the plant cell factory for secondary metabolite production. Transgenic Res. (2000) 9, 323-343.

Vuorela, H., Inhibitory activity of *Peucedanum palustre* on potassium induced contractions of rabbit aortic rings. Acta Pharm. Fenn. (1988) 97, 113-120.

Vuorela, P., Leinonen, M., Saikku, P., Tammela, P., Rauha, J.P., Wennberg, T., Vuorela, H., Natural products in the process of finding new drug candidates. Curr. Med. Chem. (2004) 11, 1375-1389.

Wagner, K.A., Yacono, P.W., Golan, D.E., Tashjian, A.H. Jr., Mechanism of spontaneous intracellular calcium fluctuations in single $GH_4C_1$ rat pituitary cells. Biochem. J. (1993) 292, 175-182.

Wang, H.S., Hwang, L.L., Sue, H.F., Lee, K.M., Chen, C.T., A simple quantitative method for evaluation of angiogenesis activity. Assay Drug Dev. Technol. (2004) 2, 31-38.

Walters, W.P., Namchuk, M., Designing screens: How to make your hits a hit. Nature Rev. Drug Discov. (2003) 2, 259-266.

Weber, G., Resolution of the fluorescence lifetimes in a heterogeneous system by phase and modulation measurements. J. Phys. Chem. (1981) 85, 949-953.

Wennberg, T., Kreander, K., Lähdevuori, M., Vuorela, H., Vuorela, P., A strategy for primary screening of natural products using micro fractionation combined with a bioassay. J. Liq. Chromatogr. Relat. Technol. (2004) 27, 2571-2590.

Wild, U.P., Holzwarth, A.R., Good, H.P., Measurement and analysis of fluorescence decay curves. Rev. Sci. Instrum. (1977) 48, 1621-1627.

Williams, M., Target validation. Curr. Opin. Pharmacol. (2003) 3, 571-577.

Withington, L., High-throughput epithelial cell culture systems for screening drug intestinal permeability. In: Cell culture models of biological barriers. Lehr, C.M. Ed. Taylor & Francis, London, (2002) pp. 94-111.

Wouters, F.S., Bastiaens, P.I.H., Fluorescence lifetime imaging of receptor tyrosine kinase activity in cells. Curr. Biol. (1999) 9, 1127-1130.

Wu, P., Brand, L., Resonance energy transfer: methods and applications. Anal. Biochem. (1994) 218, 1-13.

Wölcke, J., Ullmann, D., Miniaturized HTS technologies – uHTS. Drug Discov. Today (2001) 6, 637-646.

Zhang, J.H., Chung, T.D.Y., Oldenburg, K.R., A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. (1999) 4, 67-73.