High-Throughput Screening of Natural Products for Cancer Therapy

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Abstract

Natural products have been the biggest single source of anticancer drugs and there are continued efforts to explore the chemical diversity provided by nature in order to find new lead compounds. Bioassay test methods have developed into high throughput screening assays using both cell-based and molecular approaches. The various ways to detect effects on cell viability and cell proliferation are summarised and examples are given of developments using three-dimensional cultures and cancer stem cells. Cell-based reporter assays have also been created in order to look more directly for effects on specific physiological pathways. The molecular assays include those directed at microtubules and related proteins and at many different protein kinases.

Introduction

Natural products have been a long and continuing source of anticancer compounds [1–3]. Some of the most valuable compounds (such as paclitaxel and the *Vinca* alkaloids) were discovered serendipitously or from slow and laborious *in vivo* screening. In the last 20 years, there have been great developments in methods for both cell-based and biochemical assays so that compounds can be tested rapidly for bioactivity. Commonly available equipment allows such assays to be conducted in multiwell plates, from 96-well to much higher densities such as 1536-well and even denser formats. This approach is commonly referred to as high-throughput screening (HTS), even if the level of throughput to qualify as "high" varies from user to user. With the widespread availability of HTS facilities in industry [4] and in academia [5,6], there should be enhanced possibilities for discovering new active natural products that can form the basis for new drugs against cancer. This review will outline some of the available screening systems, highlighting those that have been used to screen collections of natural products. The screens can be used both for the detection of initial "hits" (i.e., compounds with activity against the primary target, even if potency is rather low, e.g., IC_{50} in the 5–10 µM range) and for selection of "leads" (i.e., compounds with higher activity than hits, typically 1 µM or less, and with some degree of known selectivity for the therapeutic target).

Various sources of natural products have been and are continuing to be used: plants, including those from traditional Chinese medicine [7,8], marine micro- and macroorganisms [9–12], microbial broths [13], and modified natural products [14–15]. Although there seems to be a perception that natural products (especially when used as extracts rather than single compounds) cause frequent artefacts with many assay systems, personal experience indicates that the number of false positives in a wide range of assays is no greater with natural products than with commercial screening collections of supposedly drug-like compounds (A.L. Harvey, unpublished). Where possible, screening campaigns should involve use of two different types of experimental read-out (e.g., one based on fluorescence, another based on chemiluminescence, etc.) so that compounds or extracts that interfere with one detection system can be picked out because they are unlikely to appear as active in the other detection system.

Cell-Based Assays

Cell growth/cell death

Since cancer cells are characterised, in part [16], by their ability to proliferate, an obvious approach to HTS for anticancer activity is to look for com-

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pounds that reduce growth of cancer cells in culture. Many cell lines are available that have been derived from a wide variety of human cancers, and effects of compounds on such cells can be compared to effects on non-cancerous cells to establish if there is any possible selectivity for tumour-derived cells. A recent example of the successful use of cell-based screens was with a panel of prostate cancer cells and non-cancerous prostate epithelial cell lines [17], and the marine-derived compound batzelline was shown to be selectively cytotoxic to pancreatic cells lines over the normal kidney epithelial cell line Vero [18].

There are many methods for quantifying cell growth or cell death in culture [19]: these include incorporation of radiolabelled thymidine, exclusion of trypan blue, measurement of metabolic activity, changes in ATP levels, release of intracellular enzymes, and binding of DNA-staining dyes (**Table 1**). They generally can be adapted to run on 384-well plates or even on 1536-well plates [20]. There are also methods to detect increases in apoptosis (e.g., [21,22]). However, there is no ideal detection method because test compounds can cause interference with the measurement system: for example, quenching or enhancing scintillation counts with radiolabels, interference with visible light or fluorescent signals, or inhibition of reagents (such as luciferase) in the assays (see, e.g., [23–25]). It goes without saying that all initial hits should be treated with scepticism until they have been confirmed by more extensive testing. Running different assays (e.g., one for cell viability and one for cell death) in parallel can be beneficial [26], particularly if the assays use different detection technologies (e.g., a UV-absorbance change and a chemiluminescent signal). Such assays can be run on the same wells [27, 28], and also be multiplexed with a cell-based assay to detect the apoptotic marker, caspase-3 [29].

Since cells in culture are in an artificial environment [30] and are generally growing much faster than they would *in situ*, there are concerns about the correlation of results from simple cell growth studies with those from *in vivo* experiments, and about their ability to predict activity of drugs in patients. Some attempts have been made to address this problem by making the culture environment less compatible with cell proliferation to make it more akin to the cellular environment *in vivo*, e.g., by using depleted medium and culture plates that discourage attachment and proliferation of cells [31].

Other confounding artefacts can include competition between compounds which bind to DNA and cell death markers (such as Sytox Green) that fluoresce when they bind to DNA. Metabolic indicators such as MTT and Alamar Blue can give misleading results depending on the time when the cells are sampled: damaged cells can be on the way to dying but their metabolism may be temporarily enhanced, leading to an overestimate of live cells (unpublished observations; [32]). Conversely, the MTT assay can underestimate cell numbers in conditions of high cell density, possibly because this reduces metabolic activity [33]. Nonspecific reduction of MTT to formazan can be produced by chemicals such as ascorbate and sulphydryl compounds present in culture media [34].

While assays with cell lines for cell death and proliferation are convenient for screening, there are concerns that compounds which kill readily proliferating cancer cells may not eliminate the tumour because of the persistence of cancer stem cells. Such cells are resistant to many anticancer drugs, and drug treatment may even induce their appearance [35]. There is, therefore, considerable interest in finding agents that are effective against cancer stem cells [36,37]. A problem is that such cells are normally found in very low abundance so that it is difficult to establish suitable HTS assays. One solution for breast cancer stem cells was recently demonstrated [35]. Mammary epithelial cell lines (normal and neoplastic) were enriched with cells with stem cell properties by being encouraged to undergo an epithelial to mesenchymal transition (the expression of *E*-cadherin was blocked by a short hairpin RNA). The resulting cells were resistant to doxorubicin and paclitaxel, and were suitable for HTS in a cell proliferation assay in 384-well plates. A screen of 16000 compounds revealed some that had some selectivity for the stem cell-like cells over normal cells (see results at http://chembank.broad.harvard.edu). Salinomycin, an antibiotic originally from *Streptomyces albus*, was the most active of the compounds studied in detail. Salinomycin has recently been reported to be a potent inducer of apoptosis in a wide range of cancer cells [38].

Stem cells also have an enhanced ability to form colonies when plated at low densities and to grow as spheroids (see next section). Clonogenic assays tend to be suitable only for low-throughput screening, but a soft-agar method for HTS of compounds against cells' colony forming abilities has been published recently [39]. The method relies on an automated pipetting instrument, and it has been validated with three human colon cancer cell lines: the clinically used anti-metabolite 5-fluorouracil caused concentration-dependent reductions in colony formation (as measured using Alamar Blue to estimate living cell numbers).

Table 1 Cell-based assays in use for screening for anticancer activities.

Target	Formats in use	Read-out	Comments	References
Cell proliferation and/or cell death (cancer-derived cell lines and primary tumour cells)	96-well, 384-well, 1536-well	^{3}H-thymidine uptake, trypan blue exclusion, ATP levels, metabolic activity indicators, DNA exposure, etc.	potential for interference of test substances with read-out; multiplexing recommended	[17–20, 51]
Apoptosis	as above	ELISA (cytokeratin [18]); fluorescence	as above	[21, 22, 29]
Cancer stem cells	384-well	standard cell growth assays	*E*-cadherin overexpression	[35]
Colony-forming ability	96-well	relative fluorescence between two cell layers	may detect relevant phenotypic behaviour of cancer cells	[39]
Spheroids	96-well	acid phosphatase assay; apoptosis-ELISA assay	may better mimic the *in vivo* behaviour of cancer cells	[45–47]
Reporter genes linked to molecular targets (e.g., Kruppel-like factor 5, EGFR, ubiquitin-proteosome)	96-well, 384-well	luciferase luminscence	can reveal effects on selected pathways so long as controls for off-target effects are run	[53, 54, 56, 59]
Zebrafish embryos	96-well	visual	not often high throughput	[103]

Most patients with cancer are treated with combinations of drugs in attempts to maximise therapeutic effects. Some screening methods have been adapted to look for potentially synergistic effects of anticancer agents. Combinations of two or three clinically used agents have been tested in various human cancer cell lines (e.g., [40] and in primary cultures established from human tumours [41]. There are statistically valid methods to define combinations that are synergistic in effect rather than being simply additive or even antagonistic. These methods can be used to screen for novel synergistic agents acting with standard drugs (e.g., [42]). Tests for synergism have also been applied in looking for agents that can enhance the effectiveness of the TNF family of death ligands [43,44]. In one study, 50000 compounds were screened for their ability to kill cells from a prostate cancer cell line in combination with an anti-FAS antibody [43]: eight selective compounds were identified. In another study, 16480 synthetic and natural product compounds were tested for their ability to enhance the effectiveness of the death receptor ligand TRAIL (TNF-α related apoptosis-inducing ligand) on a renal cancer cell line [44]. Initially, compounds were tested at one concentration alone and in the presence of a concentration of TRAIL that did not itself affect the viability of the cells. Hits were examined across a broad concentration range in the presence of a standard concentration of TRAIL. Eighteen confirmed synergistic compounds were found, and 14 of these were natural products, showing the value of including natural products in the compounds that were screened. Some of the synergistic compounds were effective at nanomolar concentrations. They appeared to act through a variety of mechanisms: interference with DNA, activation of caspase 8, modulation of mitochondrial membrane potential, or elsewhere in the apoptotic pathway.

Three-dimensional cultures

Monolayers of cells in culture are obviously two-dimensional whereas solid tumours are three-dimensional. Cancer cells are less sensitive to cytotoxic drugs when grown in three-dimensional aggregates, and therefore, it may be more realistic to test compounds against cells grown in some sort of three-dimensional system.

There are several methods available for growing cells in small multicellular spheroids, and there have been attempts to adapt these for HTS [45–47]. It is possible to measure drug-induced increases in apoptosis in cultured spheroids grown from several human cancer cell lines [48]. There do not appear to be any reports of spheroid cultures in HTS for testing natural product collections for potential anticancer activity.

Another approach to providing a more realistic environment for cells is to grow them on a three-dimensional matrix. A mesh of electrospun collagen microfibres was found to support colony formation by a prostate cancer bone metastatic cell line [49]. The cells in the colonies more closely resembled cells in patients in terms of their sensitivity to drugs. If it is possible to standardise the scaffolds so that large numbers of replicate cultures can be produced, then this approach could provide a more realistic drug screening platform than the conventional monolayer cultures.

Efforts are also being put into use of automated imaging techniques to track movement of cancer cells through matrices. This was demonstrated with A549 lung cancer cells grown in a collagen gel. It is suggested that such methods could be developed for drug screening [50].

Primary cells

Cell lines can show significant differences in their behaviour from tumour-derived cells in primary cell culture or xenografts. Therefore, it might be more appropriate to use primary cell cultures from biopsies obtained from patients. Then the difficulty becomes one of quantity and reliability of tissue access. Primary cultures from ovarian tumours can grow successfully in 384-well plates, thereby reducing the number of cells needed for each assay point (S. Glaysher and I.A. Cree, unpublished). This method allows HTS of compound collections for anticancer activity in primary cell cultures. In a pilot study [51], 5605 plant extracts were each screened against primary cultures established from three recurrent ovarian tumours. Sixty extracts were active, and 13 of these had previously been found to be positive in a breast cancer cell line screen (ZR75 cells) so that 47 were previously unknown from the cell line screen; work continues in order to characterise the active components in the extracts. This study shows that it is possible to use an HTS approach for large libraries of potential anticancer compounds against tumour-derived cells where previously the cell numbers required for such studies could not be achieved without use of cell lines.

In another example of the use of primary cells, B-cell chronic lymphocytic leukaemia cells were used to screen collections of compounds based on natural product scaffolds (spiroketals and fused bicyclic acetals). Potent apoptosis-inducing cytotoxic compounds were identified [52].

Cell-based reporter assays

Although simple growth and viability assays with cancer cell lines have the advantages of convenience, they do not immediately reveal insights into mechanisms of action of compounds. Mechanistic cell-based assays can, however, be created by engineering cells to express particular molecular targets (**Table 1**). For example, a transcription factor, Kruppel-like factor 5, has been found to be important for the proliferation of intestinal epithelial and colorectal cancer cells, and a suitable cancer line was stably transfected with a luciferase reporter gene to allow convenient screening for compounds that affected the expression of Kruppel-like factor 5 [53]. Wortmannin was one of the hits detected in the preliminary screening experiment, and this, and other hits, were shown to reduce growth of colorectal cancer cell lines.

An assay for compounds affecting the epidermal growth factor receptor (EGFR) was created by transfecting the receptor into a cell line (32D) so that proliferation was stimulated by EGF. Nine compounds were found to be hits out of the 20000 screened [54]. In another recent example of the development of reporter assays for detection of compounds with anticancer potential, a luciferase-based system for detecting autophagy was described [55].

Many other targets are being used in the search for novel anticancer agents, and a few are included here as illustrations. One of the most established of these targets is the ubiquitin-proteosome pathway because one inhibitor, bortezomib, is in clinical use for multiple myeloma. A bioluminescence assay that is suitable for use with natural product extracts has been developed [56] and led to the identification of physalin B from the plant *Physalis angulata* as a novel inhibitor of proteosome function [57].

There has also been considerable interest in inhibitors of heat shock protein 90 (hsp90) [58], and assays are available, both cell-based [59] and molecular [60,61]. Several natural products,

including geldanamycin and radicol, inhibit hsp90 [62]. The transcription factor hypoxia-inducible factor 1 (HIF-1) has attracted attention as a potential anticancer target. HTS assays are available (e.g., [63]), and natural products from several sources have provided some hit compounds [64,65].

There has been considerable interest in the protein interactions of p53, a tumour-suppressor gene mutated in around 50% of human cancers regulated by interaction with hMDM2 in particular [66]. Several natural product screens have been conducted, and as well as standard cellular methods, molecular methods such as phage display [67] and electrochemiluminescence methods [68]. The electrochemiluminescence method was developed to overcome some of the problems of using extracts in protein interaction assays and was used to screen a library of more than 144000 natural product extracts [68]. One natural product, sempervirine, was found to inhibit MDM2 auto-ubiquitination, MDM2-mediated p53 degradation, and led to accumulation of p53 followed by apoptosis in cells with wild-type p53.

Hits detected in such reporter assays have to be validated by showing that they are not interfering with the reporter system itself. They also need to be tested directly against the presumed target to show that they are acting through the desired mechanism. For example, a screen of marine natural products using a cell line with a luciferase reporter linked to the Wnt pathway revealed compounds that indirectly affected that pathway through inhibition of histone deacetylase (HDAC) [69]. However, an advantage of cell-based reporter assays is that the active compounds must gain access to their site of action so that an element of bioavailability is already established.

Molecular Assays

With the introduction of the so-called targeted anticancer drugs, there has been a renewal of confidence in the usefulness of assays based on molecular targets [70]. Time will tell whether such faith is justified.

Nevertheless, molecular assays do have advantages over cell-based assays: they are less laborious, they generally require less test material, they are faster, and they are more amenable to ultra-high-throughput screening (**Table 2**). However, hits from molecular assays still have to be shown to have the required selectivity for the target and to retain their activity in more integrated situations.

Tubulin and microtubule targeted agents

Given the success of taxanes (which stabilise microtubules) and *Vinca* alkaloids (which bind to tubulin and disrupt microtubules), it is not surprising that HTS methods have been developed for looking at actions on tubulin and microtubules, and on related proteins such as kinesins [71]. Novel inhibitors are being found from natural products (e.g., eleutherobin, epothilones and laulimalide [72–74]).

Protein kinases

Inhibitors of various receptor-associated tyrosine kinases have successfully reached the market as agents for specific types of cancers (e.g., [75–79]), and there are several inhibitors of cyclin-dependent kinases and inhibitors of Aurora A or polo-like kinases in clinical trials [80,81]. Since the individual kinase enzymes are available from commercial suppliers and since there are several commercially available methods for determining kinase activity [76,82], it is feasible to undertake HTS against particular kinases that are believed to be of therapeutic relevance. Not all assay formats will be ideal for use with natural products. For example, an ELISA-based assay was shown to be sensitive to antioxidants in plant extracts, whereas a time-resolved fluorescence assay was more reliable [83].

When hits are found, their specificities can be determined against a broad panel of kinases via a "kinome-wide" profiling service offered by one of several firms (see, e.g., [84]).

Examples of successful screening campaigns are those on PIM1 kinase, a serine-threonine kinase that functions as an oncogene [85] and glycogen synthase 3β (GSK3β) [86]. An ELISA-based assay for PIM1 kinase was used to screen 1200 compounds related to known kinase inhibitors, and seven confirmed hits were found. Six of these were flavonoids with IC_{50} values of 1 to 60 µM. Testing additional flavonoids led to the identification of quercetagetin, which had an IC_{50} of 0.34 µM and which was much less active against a range of other kinases. Quercetagetin was shown to inhibit the proliferation of prostate cancer cells in culture [85]. With GSK3β, analogues of the natural product staurosporine led to the design and synthesis of benzofuran-3-yl(indol-3-yl)maleimides, some of which were found to have subnanomolar potency against the enzyme [86]. Several of the compounds were potent inhibitors of growth of pancreatic tumour cell lines in culture.

Most of the kinase inhibitors developed to date bind to the ATP binding site on the enzyme, leading to concerns about achieving

Table 2 Molecular assays in use for screening for anti-cancer activities.

Target	Formats in use	Read-out	Comments	References
Kinesins	96-well	ATPase activity – colorimetric	possible interference from coloured natural products	[71]
Kinases	96-well	ELISA; time-resolved fluorescence	TRF better with natural products than ELISA	[83]
PIM1 kinase	96-well	ELISA	recombinant enzyme	[85]
p38α	96-well	fluorescence	requires fluorescently tagged enzyme	[87]
Protein-protein interactions (β-catenin/ proteosome; polo-like kinase)	384-well, 1536-well	fluorescence	robust enough for natural product screening	[89–91]
Heparanase	96-well	colorimetric	synthetic substrate	[99]
γ-Secretase	1536-well	time-resolved fluorescence	TRF may be less prone to artefacts than other systems	[100]
Leucine aminopeptidase	96-well	fluorescence	high sensitivity from choice of substrate	[101]

requisite selectivity for the targeted enzyme. Allosteric inhibitors should, in theory, be more selective, although their discovery is more difficult with conventional assay methods. An approach to finding allosteric inhibitors has been to modify the enzyme with a fluorescent tag so that conformational changes can be detected. This has been successful with the serine/threonine kinase p38alpha and with the tyrosine kinase cSrc [87,88].

Another approach to selectivity is to look for compounds that can disrupt the interactions between the kinase enzyme subunit and essential regulatory accessory proteins. Such protein-protein interactions are often regarded as "non-druggable", but this is not necessarily the case. For example, through a protein fragment complementation assay, Hashimoto et al. [89] screened over 120 000 natural product samples against different protein-protein interactions of proteins relating to β-catenin and to proteasome assembly. Several hits with IC_{50} values between 1 and 20 µM were found, and one hit from a fungal extract has an IC_{50} value of 20 nM and its complete structure is being resolved. In terms of kinases, fluorescence polarisation assays have been described for the protein binding domains of the polo-like kinases [90,91]. From a screen of 22 461 compounds, a natural product, thymoquinone, and a synthetic analogue termed poloxin were found to inhibit the protein binding domain of polo-like kinase 1 [92]. Poloxin was shown to cause mitotic arrest in HeLa cells. Similar assays for protein-protein interactions with Src kinases [93] found various plant-derived natural products to be active [94].

Other enzymes

In addition to the protein kinases, topoisomerases are well-established as targets for anticancer drugs [95,96], and HTS assays are available. Active compounds against topoisomerase 1 include the plant alkaloid camptothecin (which led to the clinically used drugs topotecan and irinotecan), and, for topoisomerase 2, etoposide (an analogue of podophyllotoxin from the mayapple *Podophyllum peltatum*) and doxorubicin (an anthracycline antibiotic related to daunomycin from *Streptomyces peucetius*).

Other enzymes being looked at as potential targets for new anticancer agents and for which HTS assays have been developed include: dual specificity phosphatases [97,98]; heparanase [99]; γ-secretase [100]; leucine aminopeptidases [101]; and histone deacetylases (HDACs) [102].

Conclusions

Molecular and cell-based high-throughput assays will continue to be developed and used to screen natural products as well as synthetic compounds for potential anticancer activity. Although many assays are available and many collections of natural products are also available, it is safe to say that not all of the compounds have been tested on all of the assays: there is still potential for new leads to be generated. Another possibility is to go beyond cell-based assays in culture and use *in vivo* phenotypic screens. Zebrafish embryos can be used in 96-well plates and, therefore, have potential for relatively high-throughput screening [103]. Cancers can be induced by exposing zebrafish embryos to chemicals such as *N*-nitrosodiethylamine, or human cancer cells can be transplanted into zebrafish embryos. If the transplanted cells have been engineered to express, e.g., green fluorescent protein, the development of the tumour cells can be conveniently monitored. Zebrafish embryos can also be genetically engineered to express human oncogenes in order to produce cancer-relevant models, and some screening of natural products is taking place [104,105].

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