In vitro assays for anticancer drug discovery—a novel approach based on engineered mammalian cell lines

Valeria Gonzalez-Nicolini[a] and Martin Fussenegger[a]

Despite decisive progress in understanding the molecular biology of cancer development, cytotoxic anticancer drugs continue to be the cornerstone of modern antitumor therapies. The developmental therapeutics program, initiated by the US National Institutes of Health's National Cancer Institute in the early 1990s, pioneered massive-scale screening for agents able to phenotypically interfere with the growth and viability of neoplastic cell lines derived from a representative panel of human carcinogenic tissues. Capitalizing on advanced knowledge of molecular processes particular for neoplastic cell characteristics, target-specific screening scenarios became since increasingly popular. With drug targets defined, natural and synthetic (combinatorial) compound/peptide/nucleic acid libraries available and the high-throughput screening technology of the systems' biology era in place, the *quo vadis* of anticancer drug discovery seems to be well determined. We review recent advances in cytotoxic anticancer drug assay design with emphasis on a novel mammalian cell-based anticancer drug finder technology for the discovery of cytotoxic drugs with fewer side-effects on non-dividing cells. *Anti-Cancer Drugs* 16:223–228 © 2005 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2005, **16**:223–228

Keywords: drug discovery, *in vitro* assays, mammalian cell lines

[a]Institute for Chemical and Bio-Engineering, Swiss Federal Institute of Technology, ETH Hoenggerberg, Zurich, Switzerland.

Correspondence to M. Fussenegger, Institute for Chemical and Bio-Engineering, Swiss Federal Institute of Technology, ETH Hoenggerberg, HCI F115, Wolfgang-Pauli-Strasse 10, 8093 Zurich, Switzerland.
Tel: +41 1 633 3448; fax: +41 1 633 1234;
e-mail: fussenegger@chem.ethz.ch

Received 8 November 2004 **Accepted** 15 November 2004

Introduction

The discovery that tumor-derived cell lines proliferate almost indefinitely promoted our present understanding of cancers, and formed the basis of pioneering *in vitro* anticancer drug discovery and testing initiatives [1]. By the early 1990s a large number of human tumor cell lines had been established and the time was ripe for extensive anticancer drug discovery in the newly available 96-well high-throughput (HTS) screening format [2,3]. A massive-scale anticancer drug screening initiative, launched by the National Cancer Institute (NCI) and the National Institutes of Health (NIH), and known as the developmental therapeutics program (DTP; http://dtp.nci.nih.gov), profiled large compound libraries for stability and solubility of individual small molecules along with their acute/chronic toxicity and proliferation inhibition on more than 60 cultured tumor-derived cell lines [4]. In current screening programs, tumor-derived cell cultures have been largely replaced by yeast mutants harboring alterations in tumor-associated genes and, by *in vitro* tests, revealing direct compound interference with key cancer targets [5] (Table 1). Although the molecular mechanisms underlying anticancer action remain elusive for most DTP-screened compounds, structure–function correlations are known for a few. Prominent examples include 5-fluorouracil (a DNA antimetabolite) [6], doxorubicin (topoisomerase II inhibitor) [7], etoposide (topoisomerase I inhibitor) [8], mitomycin C (a DNA alkylating agent) [9] and vinblastine as well as vincristine (inhibitors of microfilament turnover) [10]. All the aforementioned examples of anticancer drugs are among a set of 122 compounds whose drug action is reasonably well known for assembly as a training panel for sophisticated network analysis of drug mechanisms [11,12]. Complementary information as well as data on over 10 000 anticancer agents is available at http://dtp.nci.nih.gov. DTP also encourages submission of compounds to be evaluated for their anticancer activities.

Screening formats designed for the discovery of anticancer drugs can be grouped into cell-free and cell-based assays (Table 1 for non-limiting examples [13–27]). Recent advances in our understanding of the molecular basis of cancer development resulted in the discovery of neoplastic cell-exclusive protein targets, for which specific protein or small-molecule inhibitors are either screened *in vitro* and/or developed by rational design [28]. The aim of most prominent target-specific *in vitro* anticancer drug assays was to discover angiogenesis, metastasis and cytoskeleton polymerization inhibitors as well as apoptosis inducers [29–33]. Although promising inhibitors of all the aforementioned targets have emerged from *in vitro* screening initiatives, most of them failed in follow-up cell culture experiments due to undesired side-effects, limited specificity, stability or bioavailability [34,35].

In contrast to cell-free *in vitro* assays where drug–target interaction remains the exclusive readout, cell-based

0959-4973

Table 1 HTS-compatible anticancer drug screening assays

Assay type	Target	Readout system	Reference
Cell-free assays	serine/threonine kinase inhibitors	^{33}P incorporation	[13]
	protein phosphatase inhibitors	fluorescence	[14]
	inhibitors of microtubule formation	fluorescence	[15]
	protein kinase inhibitors	β-galactosidase complementation	[16]
	protein kinase inhibitors	fluorescence polarization	[17]
	pro-apoptotic determinants	fluorescence polarization	[18]
Cell-based assays	topoisomerase inhibitors in CHO cells	alkaline comet assay	[19]
	HepG2 hepatoma cells	GFP-based fluorescence	[20]
	apoptosis-inducing determinants in lymphocytes	colorimetric assay	[21]
	different mammalian cell lines	fluoresecence	[22]
	EGFP-engineered human cell lines	EGFP-based fluorescence	[23]
	topoisomerase inhibitors in mammalian cells	colorimetric assay	[24]
	human tumor cell lines	rhodamine-based fluorescence	[4]
	angiogenesis-inhibiting determinants in glioma cells	luciferase reporter gene	[25]
	differentiation factors (C/EBP α) in U937 cells	luciferase reporter gene	[26]
	inhibitors of the met tyrosine kinase signaling pathway in MDCK cells	chromogenic reporter	[27]

screening scenarios have the potential to score (i) intracellular target specificity, (ii) target-unrelated cytotoxicity, (iii) (metabolic) stability and (iv) bioavailability. Over 90% of drug candidates prevailing in cell-free assays fail to show any effect in follow-up cell culture assays for lack of cell penetration or metabolic conversion prior to reaching their intracellular target site [35]. While early DTP screening cell lines were molecular black boxes providing phenotypic readout limited to growth and viability profiles, latest-generation screening cell lines are transgenic and engineered for state-of-the-art ultra (u)-HTS-compatible read-out technology [36–39] (Table 1).

Cell death profiling has been by far the most popular read-out scenario of modern anticancer screening assays and a variety of different fluorescence- or chemiluminescence-based detection technologies were developed to score mammalian cell death. Examples include green fluorescent protein- and luciferase-based assays. Cell death of EGFP-transgenic reporter cells can directly be scored by HTS-compatible fluorescence quantification [40]. In another configuration, the luciferase reporter gene was placed under the control of a $SMAD_4$-specific promoter to signal tumor-suppressor pathway activation by small molecule compounds in human pancreatic cancer cells [41]. As an alternative readout to fluorescence/chemiluminescence-based monitoring technologies, dye-exclusion assays which are based on the exclusive uptake of vital dyes by dead cells have found widespread applications in screening [42–44]. For example, the use of Trypan blue staining for monitoring viability versus cell death profiles is generally accepted [45,46].

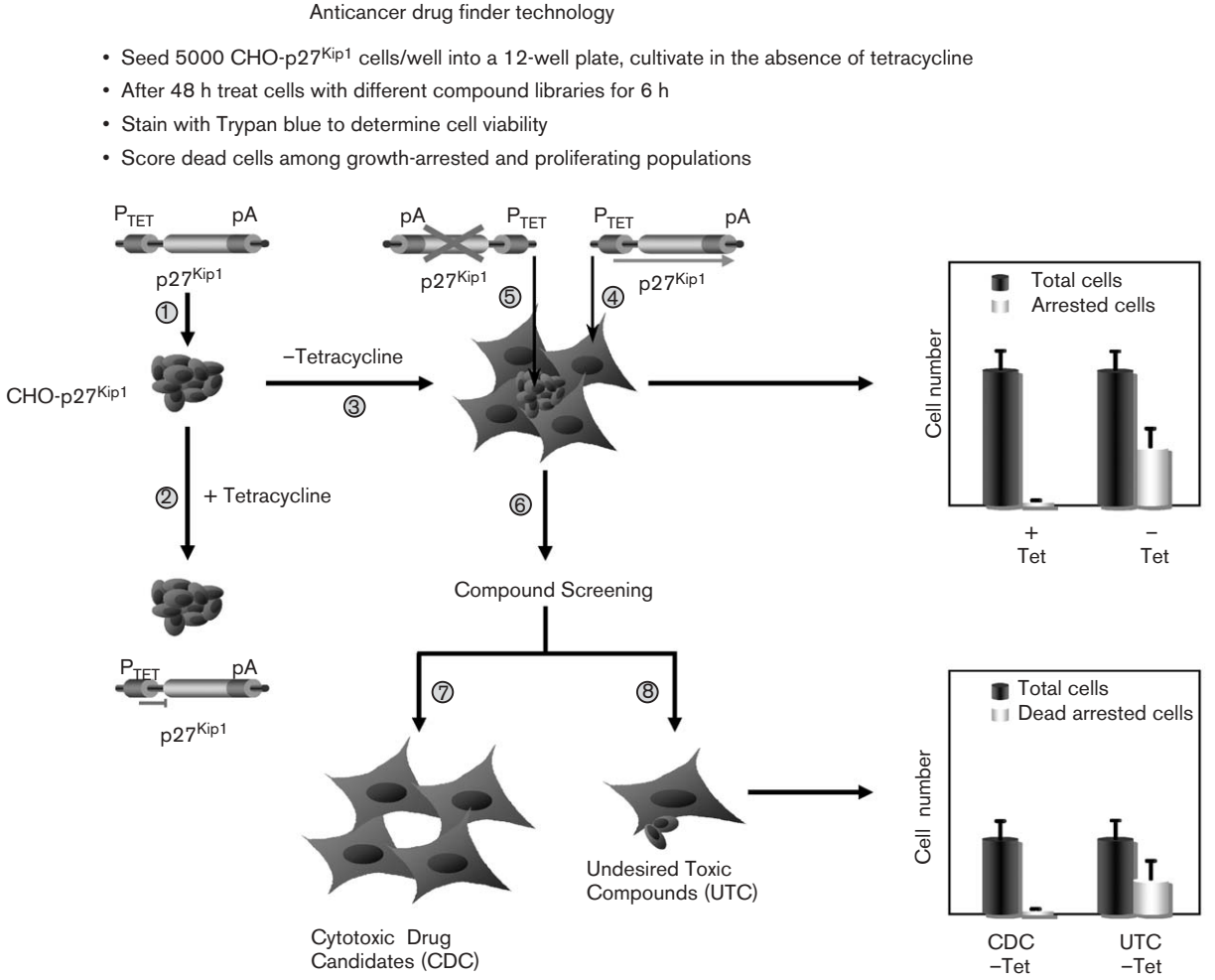
Most clinically useful drugs have been isolated from natural sources including plants and microorganisms [47–49]. Microbes like *Actinomycetes* exhibiting rapid generation times are the origin of two of the most potent anticancer drugs ever discovered: doxorubicin and actinomycin D [50]. Because of major initiatives in rational drug design, efforts on production of combinatorial chemical, peptide and antibodies libraries as well as automated synthesis of new molecule classes have been dramatically increased [51–53]. Furthermore, HTS complemented by robotic liquid handling systems and newest-generation readout systems are evolving rapidly [54–56].

We have developed a rapid and sensitive technology for detecting novel anticancer drugs. This cytotoxic drug discovery assay is based on a transgenic CHO-K1-derived cell line, which auto-differentiates into equal populations of growth-arrested (mimicking terminally differentiated body cells) and proliferating (mimicking neoplastic cells) cells (Fig. 1) [57].

Controlled proliferation technology

Genetic reprogramming of mammalian cell lines for conditional growth arrest is essential for gene therapy strategies, biopharmaceutical manufacturing, drug discovery and expansion of feeder-dependent primary cells [57–62]. Different heterologous gene regulation systems (see [63] for a review) responsive to tetracycline [58], estrogen [60] or isopropyl-β-D-thiogalactopyranoside (IPTG [62]) were engineered to reversibly control growth-arresting determinants including the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ [58,62], the differentiation factor c/ebp-α [58] or the interferon-responsive factor 1 (IRF-1 [61]). In all expression configurations arrested mammalian cells showed up to 50-fold higher specific productivity, which established this metabolic engineering strategy as controlled proliferation technology within the biopharmaceutical manufacturing community [58,64]. The pioneering controlled proliferation technology capitalizes on reprogramming cell-cycle regulatory networks by conditional tetracycline-responsive overexpression of the human cyclin-dependent kinase inhibitor $p27^{Kip1}$ in Chinese hamster ovary (CHO-K1) cells [58]. $p27^{Kip1}$ is a major

Fig. 1



Protocol and schematic representation of the anticancer drug finder technology. (1) CHO-K1 cells are engineered for tetracycline-responsive expression of the human cyclin-dependent kinase inhibitor $p27^{Kip1}$ (CHO-$p27^{Kip1}$). $p27^{Kip1}$ is driven by the tetracycline-responsive promoter ($P_{hCMV*-1}$) and terminated by a simian virus 40-derived polyadenylation site (pA). (2) When cultivated in the presence of the antibiotic tetracycline (+Tetracycline) $P_{hCMV*-1}$-$p27^{Kip1}$-pA is repressed and CHO-$p27^{Kip1}$ exhibits wild-type-like proliferation profiles. (3) Following cultivation in tetracycline-free medium (–Tetracycline), CHO-$p27^{Kip1}$ evenly differentiates into a G_1-arrested subpopulation showing high-level $P_{hCMV*-1}$-driven $p27^{Kip1}$ expression and a particular fried-egg-like cell phenotype (4), and a proliferation-competent subpopulation characterized by reproducible deletion of the $p27^{Kip1}$ expression unit and wild-type-like cell morphology (5). (6) Isogenic proliferation-competent/arrested populations are challenged with desired compound libraries and profiled for viability, e.g. using the Trypan blue dye-exclusion method. (7) Compounds with a capacity to preferentially eliminate proliferation-competent cancer-mimicking cells are classified cytotoxic drug candidates (CDC), while substances which indiscriminately kill mammalian cells are scored as undesired toxic compounds (UTC).

cell-cycle regulator, which binds and inhibits cyclin-dependent kinases managing G_1/S phase transition in mammalian cells. $p27^{Kip1}$ expression is increased in mitotically inactive terminally differentiated cells and downregulated in proliferating ones [65–69].

In its pioneering configuration, the tetracycline-responsive expression system (TET system) was derived from the *Escherichia coli Tn10* [70] and consists of the tetracycline-dependent transactivator (tTA; TetR repressor fused to the *Herpes simplex*-derived VP16 transactivation domain) and the tetracycline-responsive promoter ($P_{hCMV*-1}$; heptameric TetR operator module placed 5′ of a minimal human cytomegalovirus immediate early promoter) [71]. In the absence of tetracycline antibiotics tTA binds and activates transcription from $P_{hCMV*-1}$, while the tTA-$P_{hCMV*-1}$ interaction is abolished by tetracyclines in a dose-dependent manner resulting in graded transgene expression control (see [63] for a review; [71]).

Engineered cell-based approach to anticancer drug screening

The ability to screen for cytotoxic drugs which selectively kill proliferating cells while having minimal or no effects on mitotically inert cells remains a major goal in modern anticancer drug discovery. Currently available anticancer drug screening assays are aimed at detecting small molecules, which kill a panel of selected human cancer-derived cell lines (see DTP above). Most of the compounds prevailing in such screens show random cell-killing activities, irrespective of the cell's proliferation phenotype and/or restricted tropism for panel cell lines which result in drug candidates with limited effect or undesired side-effects [72]. We have constructed a double-transgenic CHO-K1-derived cell line (CHO-p27^{Kip1}) engineered for constitutive tTA (P_{SV40}-tTA-pA) and tTA-driven tetracycline-responsive p27^{Kip1} expression ($P_{hCMV*-1}$-p27^{Kip1}-pA) (Fig. 1). CHO-p27^{Kip1} typically expands in the presence of tetracycline when expression of cyclin-dependent kinase inhibitor remains repressed and can be switched into proliferation-controlled assay mode by cultivation in antibiotic-free media. Following cultivation for 2 days in a growth-arrested manner the engineered cell line differentiates evenly into G_1-arrested and proliferation-competent populations owing to reproducible elimination of the $P_{hCMV*-1}$-p27^{Kip1}-pA expression unit in a self-sufficient manner [57,73] (Fig. 1). Proliferation-competent CHO cell revertants lacking ectopic p27^{Kip1} expression mimic a neoplastic cell phenotype with a single well-defined mutation. Proliferation-competent/controlled cell populations can be distinguished by their cell morphology when grown in an attachment-dependent manner: while growing CHO cells exhibit a typical cell morphology, G_1-arrested cells adopt a fried-egg-like shape. p27^{Kip1}-sprecific immunofluorescence microscopy substantiated fried-egg-like cell morphology/G_1-arrest/p27^{Kip1} expression correlations [57]. Furthermore, CHO-p27^{Kip1} grown in the presence of tetracycline proliferates without any signs suggestive of G_1 arrest, indicating that the transgenic cyclin-dependent kinase inhibitor is tightly regulated.

Scoring relative viabilities of arrested/proliferating cell populations following exposure of CHO-p27^{Kip1} to chemical or metabolic libraries will reveal drug candidates with the potential to selectively kill proliferating cell phenotypes (Fig. 1).

Screening performance

In order to establish CHO-p27^{Kip1}'s potential for the discovery of anticancer drugs, we cultivated this cell line under tetracycline-free conditions and challenged it by administration of top-prescribed cancer therapeutics. Table 2 exemplifies the cytotoxic impact of 5-fluorouracil, doxorubicin, etoposide and mitomycin C treatment on CHO-p27^{Kip1} populations grown in the presence (proliferating cells only) and absence (approximately a 1:1 ratio of growing and growth-arrested cells) of tetracycline. Comparative scoring of relative viability profiles of arrested/proliferating populations indicated preferential killing of the proliferating subpopulations by established anticancer drugs. As assessed using a Trypan blue-based dye-exclusion assay, 43–61% of all cells were eliminated, which correlated with almost the entire proliferating cell population. In contrast, less viability interference on the G_1-phase-arrested subpopulation was observed: 5-fluorouracil, 13% of G_1-arrested cells, doxorubicin, 11%, etoposide, 12% and mitomycin C, 25% (Table 2). These data substantiate the selective killing of proliferating cells by clinically licensed anticancer drugs and confirm that the CHO-p27^{Kip1}-based anticancer drug finder technology is ready to apply in HTS of chemical as well as metabolic libraries to discover novel cancer therapeutics with reduced cytotoxicity on terminally differentiated cells [57]. Moreover, the anticancer drug finder technology also enables comparative analysis of established drugs or drug candidates. Table 2 shows viability profiles of CHO-p27^{Kip1} incubated in tetracycline-free media prior to treatment with a particular drug. Arrested dead cells were scored as the percentage of total dead cells, which revealed significant differences in today's top anticancer drug's with respect to undesired cytotoxicity against proliferation-inert G_1-arrested cells. For example, mitomycin C was more toxic for non-growing cells than doxorubicin. Such differences may reside in the molecular mechanisms underlying cell killing. Doxorubicin as well as etoposide inhibit topoisomerases, which eventually results in DNA damage and apoptosis. 5-fluorouracil is an antimetabolite interfering with DNA replication leading to DNA damage as well as apoptosis. However,

Table 2 Cell-death profiling of CHO-p27^{Kip1} cultivated in the presence (+) and absence (−) of tetracycline

Tetracycline		Anticancer drug (% cell death)			
		5-Fluorouracil	Doxorubicin	Etoposide	Mitomycin
+	proliferating cells (100%)	72.4	67.2	72.0	66.8
−	proliferating cells (50%)	48.8	61.0	49.4	42.7
	arrested cells (50%)	13.3	10.8	12.0	25.0

CHO-p27^{Kip1} cultivated in the presence of tetracycline form a homogeneous proliferating population (100%) since cyclin-dependent kinase inhibitor expression is repressed. CHO-p27^{Kip1} cultivated in the absence of tetracycline evenly differentiates into arrested (50%; p27^{Kip1} expressed) and proliferating (50%, p27^{Kip1} expression unit lost) populations. CHO-p27^{Kip1} cultures were treated with 5-fluorouracil (200 μg/ml), doxorubicin (800 ng/ml), etoposide (60 μg/ml) or mitomycin C (60 μg/ml). Cell death was determined using the Trypan blue dye-exclusion method.

mitomycin C-induced cell damage causes necrosis and apoptosis [9]. The anticancer drug finder technology has the potential to enable (i) detection of novel anticancer drugs, (ii) profiling of cytotoxic activities against proliferating cells as well as (iii) scoring of undesired side-effects on proliferation-inert cells. Furthermore, since human $p27^{Kip1}$ is a universal cell-cycle regulator, conditional growth control could likely be engineered into any human cell line in order to provide test and screening opportunities for novel anticancer therapeutics specific for particular cell types and tissues.

Outlook

Target-specific *in vitro* drug discovery assays provide only limited information on the potency, specificity and side-effects of drug candidates, because isolated drug targets fail to score off-target impact and miss the cellular scope of drug action. Therefore, target-specific uHTS strategies typically result in increased numbers of poorly qualified compounds being approved for expensive toxicology and animal studies where they fail at a high rate. Phenotypic cell-based assays, which offer a maximum amount of information along with first-step screening including specificity, cytotoxicity and bioavailability, are expected to increase the success rate of future drug discovery initiatives and will dramatically reduce from-discovery-to-market timelines and costs. Furthermore, it remains an ongoing challenge, associated with the treatment of polygenic diseases such as cancer, that small-molecule drugs are expected to selectively eliminate tumor cells of completely different genotypes, while leaving healthy terminally differentiated body cells unaffected. Our cell-based anticancer drug finder technology enables target-independent discovery/validation of small-molecule drugs, which selectively kill proliferating cells, a key phenotype common to all neoplastic cells irrespective of their underlying genetic profile. The CHO-$p27^{Kip1}$-based anticancer therapeutic screening and validation system is expected to be compatible with all of the currently available dye-exclusion assays and, thus, suitable for large-scale applications including DTP.

We believe that the CHO-$p27^{Kip1}$-based anticancer drug finder technology has the potential to significantly speed up the discovery and validation of highly efficient anticancer therapeutics resulting in fewer side-effects.

References

1 Holbeck S. Update on NCI *in vitro* drug screen utilities. *Eur J Cancer* 2004; **40**:785–793.
2 Finlay GJ, Baguley BC, Wilson WR. A semiautomated microculture method for investigating growth inhibitory effects of cytotoxic compounds on exponentially growing carcinoma cells. *Anal Biochem* 1984; **139**:272–277.
3 Boyd MR, Paull KD. Some practical considerations and applications of the National Cancer Institute *in vitro* anticancer drug discovery screen. *Drug Dev Res* 1995; **34**:91–109.
4 Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, *et al.* Feasibility of high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 1991; **83**:757–766.
5 Simon JA, Bedalov A. Yeast as a model system for anticancer drug discovery. *Nat Rev Cancer* 2004; **4**:481–92.
6 Geller JI, Szekely-Szucs K, Petak I, Doyle B, Houghton JA. $p21^{Cip1}$ is a critical mediator of the cytotoxic action of thymidylate synthase inhibitors in colorectal carcinoma cells. *Cancer Res* 2004; **64**:6296–6303.
7 Cipak L, Paulikova H, Novotny L, Jarosova M, Rauko P. Potentiation of doxorubicin-induced apoptosis and differentiation by indomethacin in K-562 leukemia cells. *Neoplasma* 2004; **51**:188–192.
8 Bal C, Baldeyrou B, Moz F, Lansiaux A, Colson P, Kraus-Berthier L, *et al.* Novel antitumor indenoindole derivatives targeting DNA and topoisomerase II. *Biochem Pharmacol* 2004; **68**:1911–1922.
9 Park HK, Lee KW, Choi JS, Joo CK. Mitomycin C-induced cell death in mouse lens epithelial cells. *Ophthal Res* 2002; **34**:213–219.
10 Hamel E, Covell DG. Antimitotic peptides and depsipeptides. *Curr Med Chem Anticancer Ag* 2002; **2**:19–53.
11 Weinstein JN, Kohn KW, Grever MR, Viswanadhan VN, Rubinstein LV, Monks AP, *et al.* Neural computing in cancer drug development: predicting mechanism of action. *Science* 1992; **258**:447–451.
12 van Osdol WW, Myers TG, Paull KD, Kohn KW, Weinstein JN. Use of the Kohonen self-organizing map to study the mechanisms of action of chemotherapeutic agents. *J Natl Cancer Inst* 1994; **86**:1853–1859.
13 Sorg G, Schubert HD, Buttner FH, Heilker R. Automated high throughput screening for serine kinase inhibitors using a LEADseeker™ scintillation proximity assay in the 1536-well format. *J Biomol Screen* 2002; **7**:11–19.
14 Vogt A, Cooley KA, Brisson M, Tarpley MG, Wipf P, Lazo JS. Cell-active dual specificity phosphatase inhibitors identified by high-content screening. *Chem Biol* 2003; **10**:733–742.
15 DeBonis S, Skoufias DA, Lebeau L, Lopez R, Robin G, Margolis RL, *et al.* *In vitro* screening for inhibitors of the human kinesin Eg5 with antimitotic and antitumor activities. *Mol Cancer Ther* 2004; **3**:1079–1090.
16 Vainshtein I, Silveria S, Kaul P, Rouhani R, Eglen RM, Wang J. A high-throughput, nonisotopic, competitive binding assay for kinases using nonselective inhibitor probes (ED-NSIP™). *J Biomol Screen* 2002; **7**: 507–514.
17 Seethala R, Menzel R. A fluorescence polarization competition immunoassay for tyrosine kinases. *Anal Biochem* 1998; **255**:257–262.
18 Glover CJ, Hite K, DeLosh R, Scudiero DA, Fivash MJ, Smith LR, *et al.* A high-throughput screen for identification of molecular mimics of Smac/DIABLO utilizing a fluorescence polarization assay. *Anal Biochem* 2003; **320**:157–169.
19 Godard T, Deslandes E, Sichel F, Poul JM, Gauduchon P. Detection of topoisomerase inhibitor-induced DNA strand breaks and apoptosis by the alkaline comet assay. *Mutat Res* 2002; **520**:47–56.
20 Zhu M, Fahl WE. Development of a green fluorescent protein microplate assay for the screening of chemopreventive agents. *Anal Biochem* 2000; **287**:210–217.
21 Nagami K, Kawashima Y, Kuno H, Kemi M, Matsumoto H. *In vitro* cytotoxicity assay to screen compounds for apoptosis-inducing potential on lymphocytes and neutrophils. *J Toxicol Sci* 2002; **27**:191–203.
22 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; **82**:1107–1112.
23 Steff AM, Fortin M, Arguin C, Hugo P. Detection of a decrease in green fluorescent protein for the monitoring of cell death: an assay amenable to high-throughput screening technologies. *Cytometry* 2001; **45**: 237–243.
24 Dunstan HM, Ludlow C, Goehle S, Cronk M, Szankasi P, Evans DR, *et al.* Cell-based assays for the identification of novel double-strand break-inducing agents. *J Natl Cancer Inst* 2002; **94**:88–94.
25 Rapisarda A, Uranchimeg B, Scudiero DA, Selby M, Sausville EA, Shoemaker RH, *et al.* Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. *Cancer Res* 2002; **62**:4316–4324.
26 Scudiero DA, Selby MH, Silvers TE, Laudeman J, Clopper S, *et al.* Development of a cell-based high-throughput screen for inducers of C/EBP alpha. *AACR–NCI–EORTC Int Conf*; 2001.
27 Webb CP, Hose CD, Koochekpour S, Jeffers M, Oskarsson M, *et al.* The geldanamycins are potent inhibitors of the hepatocyte growth factor/scatter factor–met-urokinase plasminogen activator–plasmin proteolytic network. *Cancer Res* 2000; **60**:342–349.
28 Bridges AJ. The epidermal growth factor receptor family of tyrosine kinases and cancer: can an atypical exemplar be a sound therapeutic target? *Curr Med Chem* 1996; **3**:211–226.
29 Constantini P, Jacotot E, Decaudin D, Kroemer G. Mitochondrion as a novel target of anticancer chemotherapy. *J Nat Cancer Inst* 2000; **92**: 1042–1053.

30 Roberge M, Cinel B, Anderson HJ, Lim L, Jiang X, Xu L, *et al.* Cell-based screen for antimitotic agents and identification of analogues of Rhizoxin, eleutherobin, and paclitaxel in natural extracts. *Cancer Res* 2000; **60**: 5052–5058.

31 Hagg M, Biven K, Ueno T, Rydlander L, Bjorklund P, Wiman KG, *et al.* A novel high-through-put assay for screening of pro-apoptotic drugs. *Invest New Drugs* 2002; **20**:253–259.

32 Paull KD, Lin CM, Malspeis L, Hamel E. Identification of a novel antimitotic agent acting at the tubulin level by computer-assisted evaluation of differential cytotoxicity data. *Cancer Res* 1992; **52**:3892–3900.

33 Roberge M, Berlinck RG, Xu L, Anderson HJ, Lim LY, Curman D, *et al.* High-Throughput assay for G_2 checkpoint inhibitors and identification of the structurally novel compound isogranulatimide. *Cancer Res* 1998; **58**:5701–5706.

34 Saijo N, Tamura T, Nishio K. Strategy for the development of novel anticancer drugs. *Cancer Chemother Pharmacol* 2003; **52**:S97–S101.

35 Corbett T, Valeriote F, LoRusso P, Polin L, Panchapor C, Pugh S, *et al. In vivo* methods for screening and preclinical testing. In: Teicher BA (editor): *Anticancer Drug Development Guide*. Totowa, NJ: Humana Press; 1997, pp. 75–99.

36 Hertzberg RP, Pope AJ. High-throughput screening: new technology for the 21st century. *Curr Opin Chem Biol* 2000; **4**:445–451.

37 Johnston PA. Cellular platforms for HTS: three case studies. *Drug Disc Today* 2002; **7**:353–363.

38 Gonzalez JE, Negulescu PA. Intracellular detection assays for high-throughput screening. *Curr Opin Biotechnol* 1998; **9**:624–631.

39 Zhu M, Fahl WE. Development of a green fluorescent protein microplate assay for the screening of chemopreventive agents. *Anal Biochem* 2000; **287**:210–217.

40 Steff AM, Fortin M, Arguin C, Hugo P. Detection of a decrease in green fluorescent protein for the monitoring of cell death: an assay amenable to high-throughput screening technologies. *Cytometry* 2001; **45**:237–243.

41 Sohn TA, Su GH, Ryu B, Yeo CJ, Kern SE. High-throughput drug screening of the DPC4 tumor-suppressor pathway in human pancreatic cancer cells. *Ann Surg* 2001; **233**:696–703.

42 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **16**:55–63.

43 Scudiero DA, Shoemaker RH, Paul KD, Monks A, Tierney S, Nofziger TH, *et al.* Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res* 1988; **48**:4827–4833.

44 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; **82**:1107–1112.

45 Allison DC, Ridolpho P. Use of a trypan blue assay to measure the deoxyribonucleic acid content and radioactive labeling of viable cells. *J Histochem Cytochem* 1980; **28**:700–703.

46 Reynolds CP, Black AT, Woody JN. Sensitive method for detecting viable cells seeded into bone marrow. *Cancer Res* 1986; **46**:5878–5881.

47 Bull AT, Ward AC, Goodfellow M. Search and discovery strategies for biotechnology: the paradigm shift. *Microbiol Mol Biol Rev* 2000; **64**:573.

48 Clark AM. Natural products as a resource for new drugs. *Pharm Res* 1996; **13**:1133.

49 Cragg GM, Newman DJ. Antineoplastic agents from natural sources: achievements and future directions. *Exp Opin Invest Drugs* 2000; **9**:2783.

50 Salas JA, Mendez C. Genetic manipulation of antitumor-agent biosynthesis to produce novel drugs. *Trends Biotechnol* 1998; **16**:475–482.

51 Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, Forrer P, *et al.* High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat Biotechnol* 2004; **22**:575–582.

52 Melkko S, Scheuermann J, Dumelin CE, Neri D. Encoded self-assembling chemical libraries. *Nat Biotechnol* 2004; **22**:568–574.

53 Robillard MS, Bacac M, van den Elst H, Flamigni A, van der Marel GA, van Boom JH, *et al.* Automated parallel solid-phase synthesis and anticancer screening of a library of peptide-tethered platinum(II) complexes. *J Comb Chem* 2003; **5**:821–825.

54 Comess KM, Schurdak ME. Affinity-based screening techniques for enhancing lead discovery. *Curr Opin Drug Disc Dev* 2004; **7**:411–416.

55 Zhou FX, Bonin J, Predki PF. Development of functional protein microarrays for drug discovery: progress and challenges. *Comb Chem High Throughput Screen* 2004; **7**:539–546.

56 Geoghegan KF, Kelly MA. Biochemical applications of mass spectrometry in pharmaceutical drug discovery. *Mass Spectrom Rev* 2004; in press.

57 Gonzalez-Nicolini V, Fux C, Fussenegger M. A novel mammalian cell-based approach for the discovery of anticancer drugs with reduced cytotoxicity on non-dividing cells. *Invest New Drugs* 2004; **3**:253–262.

58 Fussenegger M, Schlatter S, Datwyler D, Mazur X, Bailey JE. Controlled proliferation by multigene metabolic engineering enhances the productivity of Chinese hamster ovary cells. *Nat Biotechnol* 1998; **16**:468–472.

59 Fux C, Moser S, Schlatter S, Rimann M, Bailey JE, Fussenegger M. Streptogramin- and tetracycline-responsive dual regulated expression of $p27^{Kip1}$ sense and antisense enables positive and negative growth control of Chinese hamster ovary cells. *Nucleic Acids Res* 2001; **29**:19.

60 Geserick C, Bonarius HP, Kongerslev L, Hauser H, Mueller PP. Enhanced productivity during controlled proliferation of BHK cells in continuously perfused bioreactors. *Biotechnol Bioeng* 2000; **69**:266–274.

61 Schroeder K, Koschmieder S, Ottmann OG, Hoelzer D, Hauser H, Mueller PP. Coordination of cell growth in cocultures by a genetic proliferation control system. *Biotechnol Bioeng* 2002; **8**:346–352.

62 Watanabe S, Shuttleworth J, Al-Rubeai M. Regulation of cell cycle and productivity in NS0 cells by the over-expression of $p21^{CIP1}$. *Biotechnol Bioeng* 2002; **77**:1–7.

63 Weber W, Fussenegger M. Approaches for trigger-inducible viral transgene regulation in gene-based tissue engineering. *Curr Opin Biotechnol* 2004; **15**:383–391.

64 Fussenegger M, Bailey JE. Molecular regulation of cell-cycle progression and apoptosis in mammalian cells: implications for biotechnology. *Biotechnol Prog* 1999; **146**:807–833.

65 Kato JY, Matsuoka M, Polyak K, Massagué J, Sherr CJ. Cyclin AMP-induced G_1 phase arrest mediated by an inhibitor ($p27^{Kip1}$) of cyclin-dependent kinase 4 activation. *Cell* 1994; **79**:487–496.

66 Polyak K, Lee ML, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, *et al.* Cloning of $p27^{Kip1}$, a cyclin-dependent kinase I inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 1994; **78**:59–66.

67 Ladha MH, Lee KY, Upton TM, Reed MF, Ewen ME. Regulation of exit from quiescence by p27 and cyclin D1-CDK4. *Mol Cell Biol* 1998; **18**: 6605–6615.

68 Lowenheim H, Furness DN, Kil J, Zinn C, Gultig K, Fero ML, *et al.* Gene disruption of $p27^{Kip1}$ allows cell proliferation in the postnatal and adult organ of corti. *Proc Natl Acad Sci USA* 1999; **96**:4084–4088.

69 Rivard N, Boucher MJ, Asselin C, L'Allemain G. MAP kinase cascade is required for p27 downregulation and S phase entry in fibroblasts and epithelial cells. *Am J Physiol* 1999; **277**:652–664.

70 Hillen W, Klock G, Kaffenberger I, Wray LV, Reznikoff WS. Purification of the TET repressor and TET operator from the transposon *Tn10* and characterization of their interaction. *J Biol Chem* 1982; **257**:6605–6613.

71 Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 1992; **89**:5547–5551.

72 Nygren P, Larsson R. Overview of the clinical efficacy of investigational anticancer drugs. *J Intern Med* 2003; **253**:46–75.

73 Mazur X, Fussenegger M, Renner WA, Bailey JE. Higher productivity of growth-arrested Chinese hamster ovary cells expressing the cyclin-dependent kinase inhibitor p27. *Biotechnol Prog* 1998; **14**: 705–713.