Screening for apoptosis—classical and emerging techniques

Henrik Lövborg[a], Joachim Gullbo[a] and Rolf Larsson[a]

There has been a rapid development of cell-based assays and screening methods to identify promising apoptosis-inducing drug candidates for the treatment of cancer. Distinguishing between the complex processes involved in apoptosis and other forms of cell death requires information on both biochemical and morphological processes in the cell. Traditionally, many assays have been limited to measuring, for example, caspase activity using fluorogenic substrates. However, these screening assays provide only limited information on the complex processes involved in apoptosis. In this review we describe some of the available apoptosis assays amenable to high-throughput screening. In particular, image-based high-content screening assays to evaluate multiple biochemical and morphological parameters in apoptotic cells are described. Through combining the imaging of cells in microtiter plates with powerful image analysis algorithms, one can acquire deeper knowledge on multiple biochemical or morphological pathways at the single-cell level at an early stage in the development of novel anti-cancer drugs. *Anti-Cancer Drugs* 16:593–599

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[a]Department of Medical Sciences, Division of Clinical Pharmacology, University Hospital, Uppsala University, Uppsala, Sweden.

Correspondence to H. Lövborg, Clinical Pharmacology, University Hospital, Uppsala University, 751 85 Uppsala, Sweden.
Tel: +46 18 611 52 50; fax: +46 18 519237;
e-mail: henrik.lovborg@medsci.uu.se

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Introduction

During the last few decades there has been a rapid development of assays and screening methods to identify promising drug candidates for the treatment of cancer. The focus of many of today's screening assays has been to identify targeted substances affecting specific pathways in tumor cells to achieve selectivity of the developed drugs for cancer cells [1,2]. However, this approach has limitations. Most molecular targets used for potential new treatments for cancer interact with a number of other signaling pathways in the cell. Screening for inhibition of a single target is not likely to predict the efficacy of the compound in a cellular system [3]. With some exceptions, many of these attempts have failed to identify novel drugs with significant benefits in randomized clinical trials [4].

Most drugs, regardless of mechanistic class and proven effectiveness in the clinical treatment of cancer, will induce apoptosis [5–7]. Since clinically used compounds have some degree of selectivity for cancer cells versus normal cells, screening for apoptosis is a compelling alternative to target-based screening [8].

Apoptosis was originally defined by Kerr *et al.* in 1972 [9]. Since then the hallmarks of apoptosis have been nuclear and cytoplasmic condensation, nuclear fragmentation, and formation of apoptotic bodies with intact plasma membrane. Different from this active ordered mode of cell death is necrosis, usually described as a passive form of cell death with loss of plasma membrane integrity and lack of nuclear fragmentation [10,11]. However, the separation of cell death into these two categories, although often used, is an oversimplification. A specific anti-tumoral compound or toxic stimuli can induce different modes of cell death depending on concentration and schedule of administration [12,13].

According to Kerr *et al.*, the definition of apoptosis is based on morphological criteria [9]. However, over the years a number of biochemical processes important for the apoptotic morphology have been identified. Induction of proteases in the caspase family has rendered most interest during the last decade [14]. Activation of caspases is generally thought to occur either through activation of death receptor signaling or the release of apoptosis-inducing factors from the mitochondria [15]. Signaling from receptors or direct toxic stimuli on the mitochondria results in formation of pores in the outer membrane of the mitochondria. This results in loss of the mitochondrial membrane potential (MMP) and release of cytochrome *c*, subsequently activating caspases [16] leading to DNA fragmentation.

There is a strong connection between the activation of certain caspases and the morphology originally described as apoptosis [17]. However, recently there have been reports on cell death with apoptotic features without signs of caspase activation [18–20]. Apart from the caspases, the cathepsins are another class of proteases that have been studied extensively in the area of apoptosis. Cathepsins are normally localized to the

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lysosomes where they are part of the degradation of proteins. Permeabilization of the lysosomes by stress conditions or death receptor signaling can result in translocation of cathepsins into the cytoplasm. The protease activity of the cathepsins then triggers a cascade reaction resulting in cell death with apoptotic features, with or without the participation of caspases [21]. A simplified overview of some of the biochemical pathways important for apoptosis is outlined in Fig. 1.

The inclusion of biochemical characteristics into the definition of apoptosis has been discussed intensely over the past few years [22,23]. However, in this review of apoptosis screening assays we use a pragmatic definition of apoptosis. Since the definition of apoptosis is continuously evolving, we choose to cover cell-based apoptosis screening technologies based either on morphology, biochemical hallmarks or a combination of the two.

There are a number of assays for various aspects of the apoptotic process. However, many of them are not suitable for screening purposes [24]. This review is therefore not an attempt to cover the whole range of apoptosis assays, not even all screening assays. Rather this is an overview of some methods amenable to high-throughput screening (HTS) with special focus on image-based screening assays.

Traditional single-endpoint assays

Enzyme-linked immunosorbent assay (ELISA) apoptosis assays

One approach to identify biochemical markers of apoptosis is by means of ELISA. One method is based on the observation that formamide induces denaturation of DNA in apoptotic, but not necrotic, cells [25]. Following exposure to compounds, cells are fixed and the DNA of apoptotic cells is denatured with formamide. A mixture of a primary antibody directed against single-stranded DNA (ssDNA) and a secondary peroxidase-conjugated antibody is then added to quantify the degree of apoptosis. After addition of peroxidase substrate the absorbance is measured [26]. This assay was used in a small study of 13 clinically used anti-cancer drugs and 13 toxic compounds without known anti-tumoral activity using the T cell leukemia cell line MOLT-4. None of the toxic compounds indicated apoptotic processes, while all anti-tumoral drugs expressed high levels of formamide-denatured ssDNA [27]. This supports the rationale for screening for apoptosis to identify clinically useful compounds discussed earlier.

Another way of detecting apoptosis using ELISA is to monitor accumulation of cleaved caspase substrates. Cytokeratin 18 (CK18) is such a substrate present in epithelial cells [28]. An ELISA measuring the M30 epitope exposed on caspase-cleaved CK18 has been developed. This assay measures the accumulation of cleaved CK18 in culture medium as well as in cells. Cells were exposed, lysed and analyzed with the ELISA in 96-well plates allowing rapid screening of compounds. A screen of 500 compounds from the National Cancer Institute Diversity Set library identified 16 compounds that induced accumulation of cleaved CK18 [29]. One of the compounds identified as apoptosis inducing was ellipticine. Induction of apoptosis by this compound was confirmed by Annexin-V staining. One limitation of this assay is that it can only be used for epithelially derived cells. The major advantage compared to assays measuring caspase activity directly is that it measures the accumulation of cleaved CK18 over time. This eliminates the problem of identifying the optimal time-point for screening. An interesting application of this assay is the possibility of using it to monitor the effect of treatment in patients. In patients with recurrent breast cancer it was shown that those responding to treatment had significantly higher levels of cleaved CK18 in serum compared to non-responders [24].

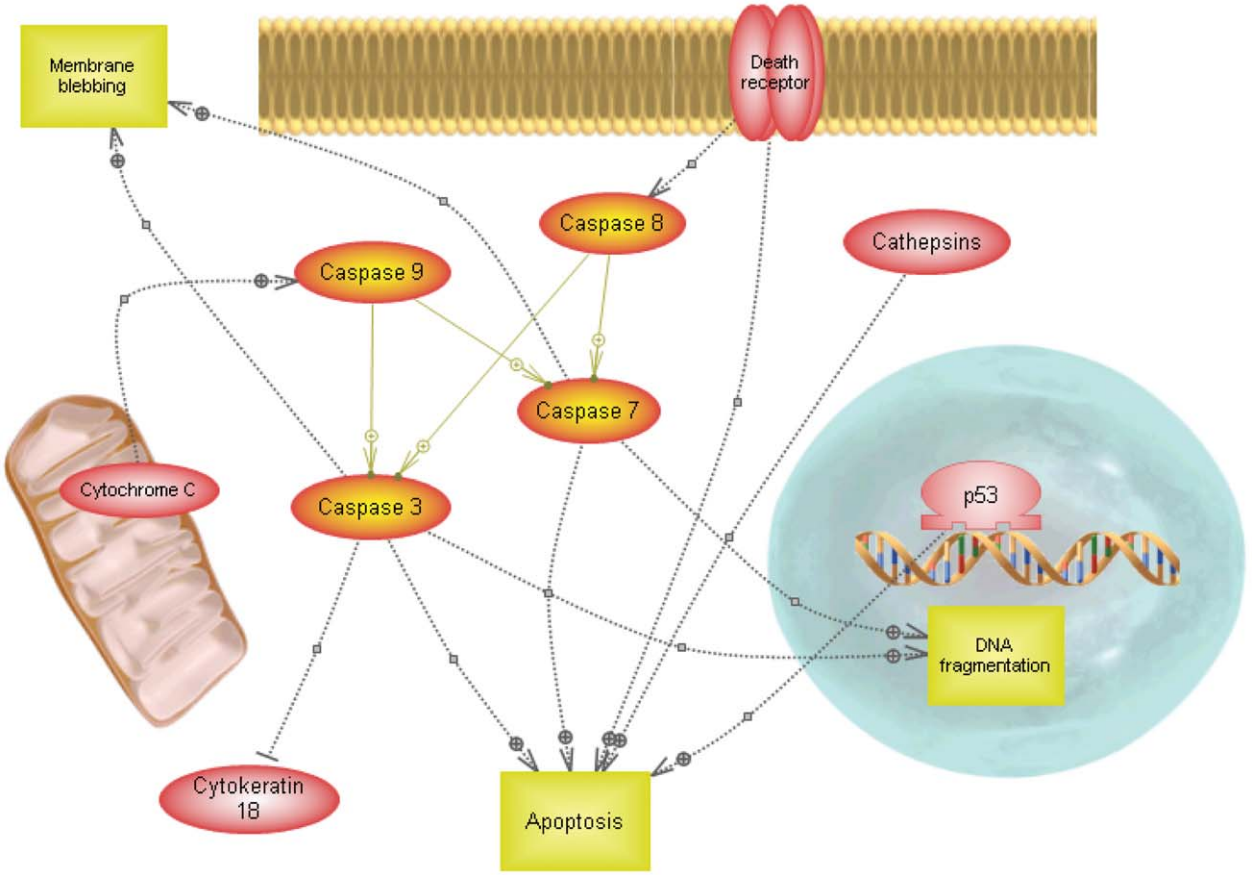
The two assays described above are both suitable for medium or HTS and as ELISA assays they rely on instrumentation that is readily available in most laboratories. However, the drawback of ELISA assays in general is that they require a number of time-consuming washing steps.

Caspase activity assays

As described earlier, the link between caspase activation and apoptosis has been the subject of intense studies. This has led to the development of a number of assays for measuring, in particular, caspase-3 activation in cellular systems. Probes based on coumarin fluorogenic substrates have been used to detect caspase-3 activity in cell lysates [30]. However, due to the need for high cell numbers per sample, high background signals and short wavelength these probes are not suitable for HTS [31]. Instead, a number of rhodamine 110 (R110)-based probes have subsequently been developed [31,32]. The principle of these probes is based on the cleavage of a caspase substrate peptide connected to R110, thereby becoming fluorescent. The advantages of R110-based probes compared to coumarin-based probes are the low fluorescence of the uncleaved probe, and the longer excitation and emission wavelengths. This results in lower background signals and less interference from fluorescent compounds in the screening [31].

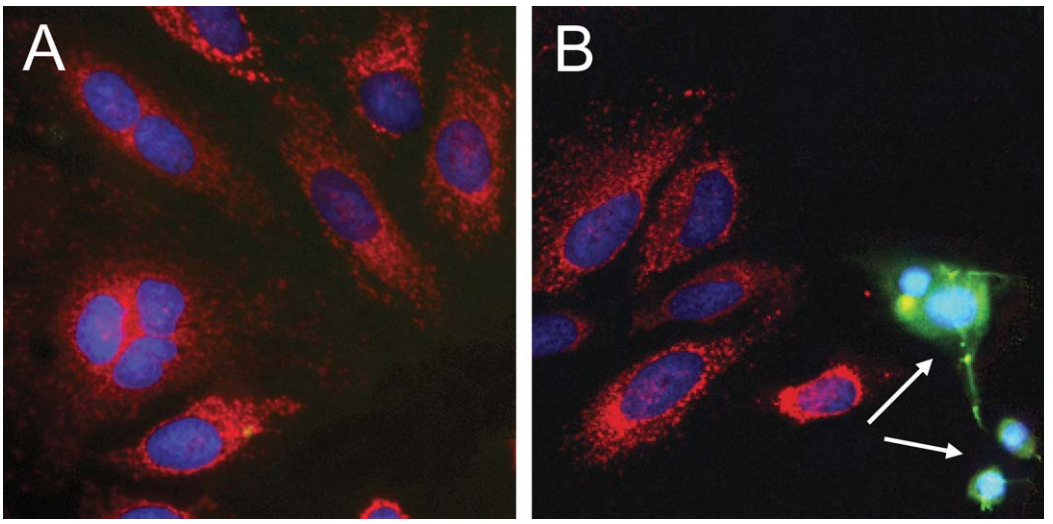
The *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110 fluorogenic substrate has been used to identify compounds inducing caspase-3 activity in cell-based HTS assays. The use of these probes allows high-capacity screening due to the convenient 'mix and read' setup. Cells are incubated in

Fig. 1



A simplified overview of pathways important for apoptosis described in this review generated using data mining and graphical presentation from the commercially available software PathwayAssist (www.ariadnegenomics.com). Processes are shown as yellow boxes and proteins in the apoptotic pathways as ovals; dotted lines indicate indirect and solid lines direct activation. References underlying the pathways described can be access by clicking on the arrows in the online version of the figure at http://www.medsci.uu.se/klinfarm/pharmacology/Figure%201.htm.

Fig. 2



Example of images from a multi-parametric HCS assay acquired using the ArrayScan HCS system. (A) Unexposed HeLa cervical carcinoma cells showing bright chloromethyl-x-rosamine staining (red) and oval-shaped, uniformly stained (blue) nuclei indicating healthy viable cells with intact MMP. (B) Exposure to 0.4 μM staurosporine for 4 h activates caspase-3 activity in some cells (green, see arrows). Note the nuclear condensation/fragmentation and reduction in MMP in cells with activated caspase-3. Automated acquisition and analysis of this type of image allows rapid quantitative measurement of multiple apoptotic features in wells as well as at the single-cell level [49].

96-well plates with compounds for 24 h followed by 3 h incubation with probe. A fluorescent plate reader is used to detect fluorescent signal in cells with active caspase-3 [33–35].

N-phenyl nicotineamides are a class of compounds identified using this assay. This class of compounds potently induced caspase-3 in the T47D breast cancer cell line. Apoptosis was confirmed by studying nuclear fragmentation using fluorescence microscopy and subsequent analysis revealed the *N*-phenyl nicotineamides to be inhibitors of microtubule polymerization *in vitro* [35]. Another apoptosis-inducing substance identified using this assay is gambogic acid [34]. Gambogic acid has also been shown to have anti-tumoral activity *in vivo* [36]. The identification of these compounds and the confirmation of morphological signs of apoptosis in a subsequent analysis strengthen the use of caspase-3-based screening for apoptosis-inducing agents.

Flow cytometric apoptosis screening

Flow cytometric analysis of apoptosis is widely used, and tools and techniques are readily available [37]. This type of analysis also allows collection of multiple-parameter data [38]. Multi-color fluorescence analysis as well as indirect morphological information from light scattering [39] makes the method a suitable basis for apoptosis studies.

However, the major limitation of flow cytometry in screening assays has been sample handling and throughput. The recent development of sample handling devices for microtiter plates connected to flow cytometers now allows sample handling rates suitable for HTS [40].

One example of a semi-automated flow cytometry-based technique for apoptosis studies has recently been described [41]. The assay is based on staining of the cell nuclei with fluorescent DNA-binding dye and simultaneous measurement of incorporation of bromodeoxyuridine (BrdU) into cells passing the S phase of the cell cycle. The measurement of DNA content allows, together with BrdU incorporation, analysis of the percentage of cells in different cell cycle phases as well as detection of apoptotic cells measured as reduced DNA content [42]. By using this technique the authors have screened a library of 1600 diverse compounds to identify compounds that enhance the anti-lymphoma activity of the clinically used antibody rituximab. Compounds were screened alone and in combination with rituximab. Twenty-two compounds resulting in more than a 200% increase in apoptosis or inhibition of the cell cycle compared to rituximab alone were selected for retesting. Fifteen of these were confirmed to enhance the activity of rituximab; among them were the clinically used drugs topotecan and aphidicolin as well as some novel experimental substances. Both topotecan and aphidicolin enhanced the apoptosis-inducing effect of rituximab in the screening assay. This effect was confirmed using Annexin-V [43] and TUNEL [44] staining. With a throughput of about 1000 samples a day in this semi-automated setting, the authors propose that flow cytometric techniques could be useful in screening arrays of compounds, concentrations and combinations for induction of apoptosis [41].

A limitation in using flow cytometry for apoptosis screening is the lack of direct morphological information. One has to rely on DNA content or granularity of the cells in combination with biochemical markers like caspase activity. It is also difficult to measure intracellular distribution of proteins in intact cells, even though attempts have been made to measure translocation of transcription factors on isolated nuclei [45].

Image-based high-content screening (HCS) assays

For researchers trying to identify modulators of specific apoptosis pathways or trying to identify general apoptosis inducers, there are a number of commercial and non-commercial compound libraries available [46]. Using libraries comprising thousands of substances, there has been a great need for automation. As described above, many of the available assays for apoptosis are now proven to be amenable to automation. However, one general problem for most apoptosis screening assays is that only limited information, on either biochemical *or* morphological parameters, is generated for each compound screened. The need for rapid information-rich screening methods is therefore increasing. Using so-called HCS methods it is possible to extract more information, including morphological data, from each well in a microtiter-based screening. Even though there is no exact definition of HCS, it normally refers to a cytometric technique based on automated fluorescence microscopy. By connecting the imaging of cells in microtiter plates with powerful image analysis algorithms and data visualization software one can acquire knowledge on multiple biochemical or morphological pathways at the single-cell level [47].

As described earlier, the events leading to apoptosis often involve the disruption of the MMP with subsequent release of apoptosis-inducing factors from the mitochondria. The released factors then induce caspase activity, eventually leading to nuclear condensation and fragmentation [48]. An assay generating data on these three important hallmarks of apoptosis, i.e. disruption of MMP, caspase activation and nuclear fragmentation, in a single screening run has recently been developed [49]. This multi-parametric HCS assay is based on imaging of fluorescently labeled cells to automatically detect

changes of MMP, caspase activity and nuclear fragmentation at the single-cell level using the ArrayScan HCS system. To probe MMP, the red fluorescent chloromethyl-x-rosamine (MitoTracker red) was used. This probe accumulates in mitochondria based on the MMP [50]. Analysis of caspase activity was done by adding fluorescent probes that covalently bind active caspases, so called fluorochrome-labeled inhibitors of caspases (FLICA) [51]. In this HCS assay a probe specific for caspase-3 and -7, FAM-DEVD-FMK, as well as a pan-caspase probe, FAM-VAD-FMK, was used to label cells with active caspases. For a measure of nuclear morphology, cells were stained with the DNA-binding dye Hoechst 33342. All steps in the assay from cell culture, exposure, staining and imaging of the cells were performed in 96-well plates. The ArrayScan HCS system allows rapid and automated fluorescent image acquisition and image analysis [52,53]. In this assay a number of images were acquired in each well to allow analysis of 500–1000 cells/well. Image analysis algorithms in the system identifies each cell, excluding artifacts, and reports on the cellular basis the fluorescence intensity of the MMP and the caspase activity as well as the size, shape and pattern of nuclear staining. Images of HeLa cells from this assay are shown in Fig. 2. Cells exposed to staurosporine show activated caspases (green), condensed nuclei (blue) as well as reduced MMP (red). This assay was shown to allow correlation of multiple apoptotic features at the single-cell level, thereby, in a single screening run, giving a more complete picture of the apoptotic process for each compound tested. Also, a number of well-studied clinically used and experimental drugs known to induce apoptosis were tested in the assay using the suspension cell line U937. Strong activation of caspase-3 was noted for etoposide, vincristine and melphalan, as well as for the experimental anti-tumoral compound CHS 828. For all drugs tested there was also a reduction in MMP, and an increase in nuclear fragmentation and condensation [49]. The advantage of this type of assays is the ability to rapidly determine multiple parameters from a single run on the well and single-cell level. At the same time the assay is useful for both deep biology studies as well as rapid screening of compounds.

Using HCS, as described above, to study apoptotic processes allows one to study the role of a specific target in the cell and correlate that to multiple apoptotic processes affected in the same cell. The tumor suppressor p53 is an important target as a trigger of apoptosis [54] and many tumors have mutations in the p53 gene. Induction of p53 due to DNA damage is known to induce arrest of the cell cycle or apoptosis [7]. This makes p53 a suitable target for analysis and screening for drug effects on the target, nuclear morphology and cell cycle distribution in a single assay. In an attempt to dissect the cellular and molecular activities of p53 and anti-tumoral and experimental drugs, a HCS assay for combinatorial cell biological studies was developed [55]. The goal of this work was to build new biological knowledge for a small panel of compounds using a multi-parametric assay on the ArrayScan HCS system and RNAi technology to modulate expression of p53. Automated immunostaining of cells was performed in 384-well plates to visualize p53, phosphorylated retinoblastoma protein, known to be an important regulator of cell cycle progression [56], and tubulin mass. Staining of nuclear DNA was used to address cell cycle distribution and nuclear morphology. By analyzing these parameters in A549 lung carcinoma cells exposed to various concentrations of a panel of *22* compounds the authors selected two compounds, camptothecin and paclitaxel, qualifying for further testing. This qualification was based on induction of p53, modulation of microtubule stabilization and cell cycle blocking activity. This selection of hits shows the ability of HCS to select compounds with complex multi-target activity in a single screening run.

Looking into the concentration-dependent processes in the cells exposed to camptothecin and paclitaxel it was possible to draw a number of important biological conclusions. Camptothecin, known to be a topoisomerase I inhibitor, was shown to induce G_2/M cell cycle block without tubulin stabilization at lower concentrations. However, at higher concentration there was a G_1/S block accompanied by stabilization of the microtubule. It was also noted that paclitaxel activated p53 only at concentrations that did not disrupt the microtubule cytoskeleton. The interesting profile of these two drugs was also followed in cells where the expression of p53 was reduced by the use of siRNA [57]. Among many other interesting findings in this study was the notion that camptothecin was ineffective as a tubulin-stabilizing agent in cells pretreated with p53 siRNA, indicating a molecular relation of the two targets [55]. Since inhibiting protein expression with siRNA is not a complete process, some cells might still express p53 to a certain degree. The advantage of HCS, in addition to the throughput and single-cell capabilities, is that expression of a target gene, e.g. p53, can be analyzed in the same cell, or subpopulation of cells, as the morphological or biochemical marker.

Recently, a multi-spectral imaging flow cytometer has been described [58]. This system brings together the benefits of image-based HCS and flow cytometry by combining six channels of bright field, dark field and fluorescent images of single cells in flow together with traditional flow cytometric analysis. By using Annexin-V and 7-AAD staining of cells it was possible to discriminate between necrotic and late apoptotic cells. In ordinary flow cytometry this is difficult since both cell populations stain positive with Annexin-V and 7-AAD. However, the morphological information from fluorescent images and

bright field images of the cells allows automated morphological discrimination between the different cell populations [58]. A clear benefit of this system is that assays and protocols developed for flow cytometry are rapidly transferred to this system, allowing additional morphological information. It also eliminates the problems present in image-based HCS with cells being out of focus and image quality problems related to microtiter plates. However, the lack of an autosampler for microtiter plates currently prevents the use of the system in a high-throughput setting. A drawback of this system and flow cytometry in general is that morphological information cannot be obtained from adherent cell lines without detaching them from their substrate.

The assays described above are important indicators of the capabilities of HCS and single-cell analysis for apoptotic studies with the possibility for HTS. However, the major challenge in these types of assays is the handling of the vast amount of data. The ongoing improvement of the image-based screening systems, especially development of software for data handling, will expand the use of the technique for apoptosis-screening purposes.

Conclusion

The methods described in this review are not a complete listing of all available assays suitable for apoptosis screening. However, it is an overview of some fundamentally different assay techniques with varying degrees of complexity and information content. Generating more information per screening well is likely to speed up the process as well as limiting the number of false hits. It is likely that the need for multi-parameter screening assays, in different areas of drug discovery and development, will shift HTS into HCS [59]. The use of image- or flow-based HCS techniques will hopefully generate better hits or even identify compounds that were not found interesting in conventional assays [60]. Whether these screening techniques will result in more qualified candidate substances for clinical trials in cancer patients remains to be seen.

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