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A Novel Reporter System for Molecular Imaging and High Throughput Screening of Anticancer Drugs

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Abstract

Apoptosis is irreversible programmed cell death characterized by a cellular cascade activation of caspase-3, which subsequently degrades proteins and other components of cells with a motif sequence. Here we report a novel reporter system to detect apoptosis, growth arrest and cell death based on controlled and self-amplified protein degradation. The key element of the reporter system is an apoptotic sensor chimerical protein which consists of three components, the procaspase 3, ubiquitin (Ub), and a strong consensus sequence of N-degron. Between each of these units, there is a DEVD (Asp-Glu-Val-Asp) sequence, which acts as the cleavage target of caspase-3. This non-conventional signal loss approach is much more sensitive than other native methods based on signal gain. The superior sensitivity is demonstrated by its effective application in 386-well HTS with lower drug concentrations and short incubation time. The HTS selection process using this reporter system is very simple and economic. The simplicity eliminates potential errors introduced by multiple steps; there is no need for any substrate. Furthermore the cells in the assay need not be disrupted and the morphology of the cells can provide additional information on mechanisms; the intact cells after HTS can also be used for other analytic analysis. This system thus has a potentially important role in the discovery and development of new anticancer drugs, it also appears to be very versatile, can be used both in vitro and in vivo, with different linked reporter genes, it can be used for a variety of imaging applications.

Keywords

Apoptosis; HTS; Drug discovery; Caspase; Molecular imaging

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Introduction

Apoptosis is programmed cell death and involves a predictable sequence of structural and biochemical changes through two pathways. The extrinsic pathway is induced by signaling via cell surface binding of death activator proteins. The intrinsic pathway is triggered by signals from inside the cell, such as those induced by damage, starvation or disturbances in the cell cycle. Both pathways converge into a cellular cascade activation of caspase-3, which subsequently degrades proteins and other components of cells with a motif sequence DEVD. Because caspase activation is an early event in irreversible apoptosis, an effective reporter system to detect apoptosis could have a significant impact for the development and evaluation of effectiveness of new cancer drugs. Currently, caspase activity can be directly measured in cells can be achieved by several reliable methods. Commercial detection kits are based on an analog substrate with a pair of quenched fluorophores linked by a DEVD peptide, which generally require disruption of the cells to release caspase for interaction with the substrates. Membrane penetrable fluorophores have also been developed for in vivo application,^[1] as well as fluorescent activity-based probes that covalently label active caspases in vivo.^[2] These and similar approaches each consume a costly substrate. Instead of using chemical fluorophores, methods based on fluorescence resonance energy transfer (FRET) between two fluorescent proteins linked by DEVD^[3] or using an inactivated luciferase precursor^[4] have also been devised. However, considering that apoptosis will eventually lead to cell death, and thus slow down or turn off all the transcriptional and translation machinery, the decrease of reporter protein resulting from reduced new protein synthesis as well as decreased cell numbers (less proliferation and/or cell death) will offset or even cancel the signal gain converted by caspase. Here we describe a novel reporter system which requires no substrate. It also appears to be more versatile than previously described systems and sensitive enough for high throughput screening (HTS) applications. It can be used both in vitro and in vivo; by linking with different linked reporter genes, it can be easily adopted for optical, PET and MRI imaging.

Results

Construction of reporter

Most current detection systems are designed to be “turn-on” by caspase, but the signal gains in these approaches will be offset by the loss of reporter protein and whole cells in the progression of apoptosis. In addition, the signal gain is interfered by universal but varieties of background signal/noise associated with cells, Therefore these approaches need to establish a predetermined time window to obtain the optimal signal; generally the time of this window is narrow and drug dependent. These constraints also apply to the assays based on measuring caspase activity directly. To eliminate these undesirable behaviors, our reporter system was designed to behave in an opposite manner, so that the cells are initially ‘light-up” but then turned off by apoptosis. Protein degradation, cell arrest and death will all contribute to the signal reduction, especially compared to the signal from the untreated control cells, in which the signal will increase exponentially with cell proliferation. Therefore the difference between treated and untreated cells increases over time, and the time window is not critical. The light-on state itself may also greatly facilitate the measurement of tumor growth and the tracking of metastasis (see Supplementary Fig. 1). As illustrated in Figure 1B, the key element of the reporter system is an apoptotic sensor chimerical protein which consists of three components: the procaspase-3, ubiquitin, and a strong consensus sequence of N-degron, separated by a DEVD sequence. In response to the apoptotic signal, the cleavage at DEVD sites by activated caspase-3 will generate two unstable c-terminal portions of proteins with the reporter in the c- end. One has ubiquitin in the N-terminal; the second one has N-end rule (or N-degron) sequences. In eukaryotes, linear Ub-protein fusions are rapidly cleaved by Ub-specific protease (UBPs) at the Ub-

protein junction. [5, 6] Thus the last amino acid glycine (G) of ubiquitin was replaced with two valines (V), and this forces the N-terminal ubiquitinated protein to be degraded by the proteasome, [7] but C-terminal linked ubiquitin will be removed as usual (such as, if cleavage occurs at the second DEVD site, pre-caspases-3 fused to ubiquitin will be deubiquitinated quickly). The second product from potential cleavage contains an N-degron which relates the in vivo half-life of a protein to the identity of its N-terminal residue. Various forms of the N-end rule operate in all organisms. In eukaryotes, the N-end rule pathway is a part of the ubiquitin system. N-degron is the most powerful degradation signal. Details of the N-end rule and its pathway have been well characterized. [8, 9]

The tagged proteins will be degraded and result in signal reduction. In addition, the cleavage of DEVD will also release the N-terminal procaspase 3, which initially acts as a protection cap for the stabilization of the whole fusion protein. This will be further processed and converted to active caspase-3, which will act back on the fusion proteins. This feedback loop will generate more active caspase-3, which will also act on other cellular targets to accelerate the apoptotic process and eventual cell death. All these factors contribute to the overall reduction of reporter protein or the disappearance of reporter signal. Since it is hard to predict the accessibility of the DEVD sequence in a fusion protein, two DEVD sites were inserted in two different locations to increase the chance hit by caspases. Secondary structure analysis shows that both DEVD sites in the fusion protein have relative high hydrophilicity, and predict that the first DEVD is in the middle of a coiled β -strand, while the second DEVD may form a β -helix surrounded with coiled structures. To enhance the expression of cloned genes and facilitate the establishment of stable cell lines, the whole open reading frame of the chimerical protein was moved into an F721 vector. [10] The final constructed plasmid is shown in Fig. 1A, and the designed chimerical protein is named as sensor-EGFP, the control vector without the “sensor” conjugation to express EGFP alone was also constructed.

Characterization of the expressed reporter proteins

The reporter proteins showed robust expression in most cell lines due to the presence of the elongation factor alpha promoter and intron sequence. To confirm the expression and right components of the designed proteins, whole cell lysates (WCL) were extracted from C6 cells stably transfected with vectors expressing EGFP (lane 1 of Fig. 2A) or Sensor-EGFP (lane 2 of Fig. 2A). WCL proteins were separated by SDS-PAGE and blotted with antibody against either caspase 3 (left panel) or GFP (right panel). Both antibodies show a fusion protein with molecular weight (MW) around the predicted 75kd. Noticeably, both antibodies show several smaller protein bands, likely partially degraded products or alternatively translated proteins.

Utilization of IRES greatly reduces false positive clones. As shown in Fig. 2B, analysis by fluorescent activated cell sorting demonstrated more than 99% of drug (puromycin) resistant cells are positive for both EGFP alone and for sensor-EGFP fusion. By considering that most mammalian cells are highly sensitive to puromycin, the combination of IRES and puromycin as selection antibiotics greatly accelerates the introduction of the designed fusion protein into most cells. As also be demonstrated by the results in Fig. 2B, the sensor-EGFP fusion (middle panel) produces (about 50%) less optical signal compared to cells transfected with EGFP alone (right panel). The weakness of the signal strength in the sensor-EGFP fusion may result from the low level of protein expression and/or because the fused protein has slightly different folding, which leads to a lower fluorescence quantum yield. Nevertheless, the fluorescence from the sensor-EGFP fusion is still very bright, and can be easily detected by microscope or a plate reader for as few as several thousand cells.

Confocal microscopy reveals that EGFP alone localized throughout cells including the nucleus (left panel of Fig. 2C) in C6 rat glioma cells. The small size of EGFP allows it enter into nuclei by passive diffusion through nuclear pores. In contrast, the sensor-EGFP fusion is excluded from the nuclei and distributes mostly in cytoplasm and shows a more granular distribution (right panel of Fig. 2C). This pattern is consistent with the localization of procaspase-3 in cytosol and mitochondria in various rat tissues. The amount of procaspase-3 in mitochondrial has been reported to be correlated with the likelihood of spontaneous apoptosis.^[11] Elevated expression of procaspase-3 had been reported for tumor cells, over expression of either procaspase-3 or caspase-3 was found not lead to the apoptosis of cells.^[12, 13] In our reporter system, procaspase-3 is expressed as a part of a larger fusion protein; this will alleviate the self-activation of procaspase-3, and subsequent effects on the phenotype of cells. Indeed our data show the expression of fusion protein increased the background caspase activities 2 to 4 folds, but had minimal effect on the C6 cells' growth (the cell proliferations were determined by CyQuant to measure total DNA; after 3 days' growth, both cell lines showed less than 5% difference). The effects of the reporter integration on the caspase activities were also tested at the presence of different chemical compounds for both C6 and DLD1 cell lines; the results show that the chemical compounds tested (10 μ M CPT, 100 μ M ZnCl₂, 20 μ M PBR06 and 2 μ g/ml Cycloheximide, see below section for description) all increase about the same folds of caspase activity as they did to the corresponding non-reporter-transfected parental cells after 24 hours treatment. This suggests that it's unlikely the reporter will interferes with the endogenous apoptotic machinery; but it may amplify and enhance candidate drug's potential.

Drugs that induce apoptosis reduce the fluorescent signals of the reporter system

To examine the fluorescence change induced by apoptosis, C6 cells transfected with EGFP and sensor-EGFP were treated with 10 μ M of camptothecin (CPT), a potent inhibitor of topoisomerase I, which has been shown to induce apoptosis in a dose-dependent manner in vitro. The fact that CPT is capable of inducing apoptosis in C6 cells has also been reported.^[14] After 3 days' treatment, the cells were observed under a fluorescence microscope. Fig. 3A shows the results for cells expressing sensor-EGFP in which both cell numbers and fluorescence signals were notably decreased. In addition, the distribution of fluorescence also changed; and was found in the nucleus. This phenomenon may be caused by EGFP without linked sensor from alternative translation; or diffusion into the nucleus by partially degraded smaller fusion proteins; or the redistribution of partially cleaved procaspase-3 fusion proteins. In addition to cytosol and mitochondrial localization, active caspases-3 was also found in the nuclear fraction. The selective localization of caspases in different subcellular compartments has been suggested to play an important role in their activation. The translocation of active caspases to other subcellular compartments appears to be critical for the development of the apoptotic process.^[15] Visual observations under the microscope suggest that the fluorescence signal behaves as expected from the original design. To test how the reporter system responds to treatment with various compounds, changes of fluorescent intensity were measured after being treated with PRB06, Bortezomib, Cycloheximide and ZnCl₂ in a 96-well plate. As show in Fig. 3B, all four compounds decrease the signals of cells transfected with sensor-reporter system, compared to vehicle control(see dark colored bars); and the changes are very significant and readily detectable. PBR06 (N-fluoroacetyl-N-(2,5-dimethoxybenzyl)-2-phenoxyaniline) is a high affinity ligand for the translocator protein (TSPO, also referred to as peripheral benzodiazepine receptor – PBR).^[16] TSPO is a component of a mitochondrial permeability transition (MPT)-pore, and plays a critical role in the mechanism of apoptosis. Specific TSPO ligands can induce apoptosis in rat C6 glioma cells.^[17] In our experiment, 20 μ M of PBR06 caused more than 40% florescence signal reduction, but interestingly, it had minimal, non-detectable effect on cells expressing EGFP alone (see light colored bars). PBR06 has about the same pro-

apoptotic effects as 100 μ M ZnCl₂ used in our experiment. Low level Zn was reported to inhibit the conversion of procaspase into active caspase-3^[18] and protected against programmed cell death induced by heavy metals, but elevated extracellular levels of zinc alone caused programmed cell death in C6 cells.^[19] We observed that 100 μ M ZnCl₂ can effectively arrest C6 cell growth. Cycloheximide had the same dramatic effect on the signals of cells expressing EGFP alone or cells expressing the sensor-EGFP reporter at a low concentration of 2 μ g/ml. Cycloheximide is a protein biosynthesis inhibitor and exerts its effect by blocking translational elongation. It has been reported that cycloheximide mediates apoptosis in human glioma cells via the CD95-dependent killing pathway.^[20] In addition to inducing apoptosis, cycloheximide blocks the synthesis of reporter protein; these two factors together may contribute to the rapid disappearance of fluorescence signal. The last compound tested was Bortezomib, a N-protected dipeptide type of proteasome inhibitor used clinically for treating certain type of cancers such as multiple myeloma.^[21] Since our reporter sensor utilized the protein degradation through the ubiquitin proteasome system, this system may not be able to detect this class of inhibitors because the potential artificial stabilization of the reporter protein, the results show that Bortezomib slightly stabilized the signal from the cells expressing sensor-EGFP (compare to cells expressing EGFP alone, see Fig. 3B), this can be caused by the expected stabilize effect of inhibiting proteasomes and/or the fused sensor proteins partially neutralized the effects of Bortezomib, but the overall signals were still dramatically reduced, so Bortezomib caused cell arrest and death made a much bigger contribution to the reporter signals. Thus, these data suggest that this reporter system can detect the effects of various compounds regardless of the underlying mechanisms.

The EGFP-expressing vectors not only provide the control or reference to the sensor reporter vector, itself can be effectively used to assess cell proliferation. C6 cell growth studies show the reporter fluorescence signal perfectly overlap with the data obtained by quantifying the DNA content with CyQuant assay kit (data not shown), therefore the EGFP-expressing vector provides an alternative or better tool (compare to various versions of MTT assays) for assessing cell titer. The sensor reporter can detect all the change as EGFP-expressing vector can do, but more (see Fig. 3B). For comparison purpose, we also examined the effects of various compounds on C6 cells at different time points assessed by our reporter system and commercial assay kit, the assay kit we selected was a representative of nonradioactive cell proliferation assays based on the spectrophotometric detection of tetrazolium salts (MTT), there are many versions of modified forms of MTT assay,^[22] Clontech's WST-1 is one of the relatively simple ones by using water soluble MTT and preserving the live cells. The data from direct plate reading by using the reporter system is presented in Fig. 3C, and data obtained by using WST-1 is summarized in Fig. 3D. In addition to the advantage of no extra cost and brief assay procedure (no steps to add reagent and incubation time, etc), the reporter system demonstrates the same or higher sensitivity and better data quality (smaller error bars), the reference control for reporter system had a longer linearity (reaches saturation more slowly) and therefore offers a wider range of detection window. It can also detect some effects (like PBR06) not reflected by simple cell numbers.

The reporter system can be effectively used in high throughput screening

The large effects on the readout in 96-well plates suggest that this system should also work with a 384-well plate format. To test the reproducibility and robustness of the assay, the variances of two cell lines treated with or without 10 μ M CPT were determined. Fig. 4A shows the results. The calculated z factor, which quantifies the suitability of a particular assay for use in a full-scale high-throughput screen,^[23] was 0.59 and 0.61 for C6 and DLD1 cells respectively. A z-factor of 0.5 is equivalent to a separation of 12 standard deviations, so our higher values confirm the robustness of our reporter system for the identification of chemical compounds with cancer-fighting properties.

As a proof-of-principle for application in high-throughput screening, the NCC library was screened against glioma and colorectal cancer cells concurrently. The NCC is a small library of approximately 480 small molecules that have a history of use in human clinically. The hits identified by screening the NCC library with 10 μ M concentration are summarized in Table I. There were 28 hits for the two cell lines, and the degree of inhibition for those compounds for C6 cells are plotted in Fig. 4B where hits are shown as the spots on or below the dot line. Sixteen hits are known anticancer drugs targeted to DNA synthesis and mitosis. Eight hits were reported having anti-cancer activity, or are on trial as cancer drugs. Among them, two are members of the statin family (Mevastatin and Itavastatin, developed as cholesterol lowering medicines);^[24, 25] and one is Itraconazole (an antifungal compound) which has been reported to inhibit cancer growth through the hedgehog pathway.^[26] Three hits seem not directly related to anti-cancer, one of them, diphenylcyclopropenone, an irritant, is used as a hair growing medicine, and has been reported to be able to treat extensive cutaneous metastatic melanoma.^[27] Its inhibitory effect on tumor cells may simply result from its general toxicity. Another hit, piribedil, the antiparkinsonian drug, preferably inhibits the growth of C6 over DLD1, possibly suggesting that proliferation of glial cells contribute to the pathogenesis of Parkinson's disease. The last one, nisoldipine, a calcium channel blocker used in treating high blood pressure, inhibited both C6 and DLD1 cell growth in our screening, but there are no previous reports about its anticancer capability. One hit (Idarubicin) is a strong fluorochrome and can be up-taken by cells easily, thereby producing a very strong signal. This drug did inhibit cell proliferation as determined by examination under microscope. The read-out from our reporter system will be misleading if the tested compound is fluorescent in nature. However, one of the advantages of this reporter system is that cells are still intact after the assay, so they can be examined under a microscope for visual verification. The fluorescence images can also provide clues about a compound's mechanisms of action based on the cell morphology and intracellular structure. Images from three hit drugs are shown in the Fig. 5A and 5B for each cell lines. The size, morphology, fluorescence distribution and strength showed distinct patterns for each hit. This reporter system can also directly be used for the determination of dose-dependent responses without any reagents and extra steps. Several selective hits were analyzed and the IC₅₀ was calculated. One example is etoposide, as shown in the Fig. 5C, for which the calculated IC₅₀ for C6 rat glioma cells is 0.2 μ M, close to the 0.4 μ M reported for human cells.^[28]

The pilot screening was performed at 10 μ M compound concentration for 48 hours treatment to demonstrate the sensitivity and effectiveness (it will get more sensitive for more commonly used 20 μ M and 3 days treatment in HTS). It produced very low (close to zero) false positive, but it missed some antineoplastic compounds in the library, possibly because of the low concentrations we used, or the cell lines we used are relatively resistant to those compounds, or the drug incubation time is not long enough.

Discussion

Since the advances of molecular genetic analysis from 1970s, drug discovery by HTS has increasingly embraced target-based approaches. Drug targets are typically proteins that play an essential role in disease pathogenesis.^[29] The advantage of the target-based approach include the ability to apply molecular and chemical knowledge to investigate specific molecular hypotheses, and to utilize both small-molecule high-throughput screening strategies and biologic based approaches, such as identifying monoclonal antibodies.^[30] However, despite the popularity, the target-centric approach has delivered the lowest rate of new drug approvals in generations.^[31] The poor productivity was confirmed by a recent study showed the contribution of target-based screening to first-in-class small molecule drug discovery is far less than those contributed by systematic or phenotypic approaches (17 vs.

28).^[30] Because it's difficult to fully recognize disease relevant targets, often a hypothesis was formed around biased ideas, and the targets were selected based on available, but incomplete knowledge. Furthermore, tumorigenesis not always results from a single static target, the triggering or key target may subdue to other targets with the advance of tumor. Therefore there is a compelling rationale for alternative approaches to improve screening procedure and increase success rate.^[32] Our pilot screening data demonstrated the effectiveness of this cell-based reporter system in HTS applications. This system does not bear the bias limitation of target-centric approach, and detects compounds that lead to cell arrest and death, irrespective of the precise mechanisms or targets, but preferably enhance the signal caused by pro-apoptotic drugs. By comparing screens on two or more cancer cell lines at the same time, it can select tumor specific drugs. For example, from the NCC library, Trileptal, which used in treating brain tumors, was found highly effective on C6 glioma but had less effect on DLD1.

The pro-apoptotic hits can be selected out by comparing the signal reduction to a non-sensor conjugated reporter. This can be done by measuring signals simultaneously from two fluorophores such as sensor-EGFP and mCherry alone. The difference of the percentage of signal decrease between the reporter systems (with sensor, green channel) and mCherry (red channel) can be defined as the contribution from apoptotic machinery. The preferred method for large scale HTS screening is two step measurements by using cells individually transfected with sensor-EGFP fusion or EGFP alone. This strategy is what we used here to characterize this system. In this approach, the preliminary candidate selected from the reporter screening are tested on the same, but EGFP alone transfected cells. By determining and comparing the difference of signal reduction with this setup at several time points, it can also help to figure out if a pro-apoptotic drug activates caspase directly or indirectly. Although caspase activation is an early event of irreversible apoptosis, but caspase activation not necessarily results in apoptosis or cell death. Our recent screening found one reported caspase inhibitor has great anticancer potential (data not shown), this suggest the caspase activity is an essential process of cell survival. This reporter system will preferentially select out drugs that caused cell death by elevating caspase activity over those simply elevating caspase activity. Methodologies and strategies can also be developed to refine those candidates that show inhibitory effects on both cell lines to select novel drugs.

Overall, our signal loss approach is much more sensitive than other native methods based on signal gain; our approach expands both top and bottom boundaries to close to the theoretic limits. The top boundary is expanded exponentially with cell growth, in addition to the high level expression of reporter gene (which is driven by one of the strongest promoters), the bottom boundary is approaching zero faster with multiple factors including the caspase feedback activation loop. So there is a big range to detect drug's effect in our system. The superior sensitivity is clearly demonstrated by its effective application in 386-well HTS with lower drug concentrations and short incubation time. The HTS selection process using this reporter system is very simple and economic. It only needs seeding the cells, adding compounds, and then reading the plates. The simplicity eliminates potential errors introduced by multiple steps; there is no need for substrates, and the procedures save on supplies and manpower. Furthermore the cells in the assay need not be disrupted and the morphology of the cells can provide additional information on mechanisms; the intact cells after HTS can also be used for other analytic analysis.

Despite the advantageous features of the reporter system in HTS, at this point, it's not clear whether this type of screening can dissect out molecules that preferentially target cancer over normal primary proliferating cells. There are also several potential pitfalls. For example, the transfected reporter may sensitize the cells, and could exaggerate the effect of compounds on the cells, or it may even interferes with the endogenous apoptotic machinery

under some extreme situations, so this system may not work well for cell lines that have high endogenous caspase-3 activities. Our data show various tumor cells exhibit at least 10 fold differences in their endogenous caspase activities. Reporter transfected cells did show elevated endogenous caspase activity, but it apparently did not affect the growth of cells, possibly because the expressed reporter proteins act as a competitive substrate for caspase to compensate the elevated activity. If a cell line can be stably transfected with the reporter system, it may be assumed the endogenous caspase-3 activity is not a concern for the tested cell line. To deal with suspension cells, such as leukemia cells, an extra step to spin down the cells is required for HTS. Although phenol red free media alone was found to not produce interfering signal, serum proteins show an unacceptable background, and so it is necessary to remove media from the samples before plate reading. Also this method is based on fluorescence, so any other fluorescence sources, such as auto fluorescence from dead cells, or from a compound itself, or a metabolite, will interfere with the assay. However the likelihood that auto-fluorescence of a hit compound and the decrease caused by the drug will exactly cancel each other, is very small. For unusually high readings, the true effect of compound can be determined by examining the cells under fluorescent microscope.

This reporter concept can also be extended to other applications by substituting the reporter protein. For example, when GFP is substituted with mCherry, this reporter system can be used in optical imaging of xenografted small animal models. Such as the reporter integrated C6 cells can be implanted in the rat brain to form a tumor, then using optical imaging system to test the therapeutic efficacy of drugs non-invasively. Since the reporters reside inside each cell uniformly, the optical or microscopic images can reflect the changes in the microenvironment without performing dissection or staining. The reporter can also be an enzymes, such as luciferase for bioluminescence imaging.^[33] If herpes simplex virus thymidine kinase is linked as the reporter, it may be used for positron emission tomography (PET) imaging.^[34] Finally, the reporter can also be just an artificial peptide, not a functional or structure protein, so it may be adapted as a CEST (Chemical Exchange Saturation Transfer) probe^[35] for MRI imaging. Moreover, in principle, the consensus sequence of DEVD for caspase-3 cleavage sites can be substituted with different sequences to detect other proteases. Considering the simplicity and effectiveness of this reporter system, it may significantly impact the process of drug discovery as well as the evaluation of therapeutic efficacy in vivo.

Experimental Section

Cell culture

Rat glioma C6 cells and human colon cancer DLD1, SW620 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM medium supplemented with 10% FBS and 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen, CA) under standard culture conditions in a humidified incubator maintained at 5% CO₂ and 37°C.

Plasmid construction

mCherry cDNA and pEGFP vectors were obtained from Clontech (Mountain View, CA). With pEGFP-N1 vector as backbone, a chimerical fusion protein was constructed step-by-step. First, cDNA that encodes for ubiquitin (without the start ATG) and the sequence of N-degron (also called N-end rule with amino acid sequence RHKSGAWKLPVSLVK) separated by the DEVD encoding sequence was synthesized with an overhang in the N-terminal with a designed PstI, and a C-terminal SalI digestion site, and the whole cDNA was cloned into a pSMART vector. To prevent the removal of ubiquitin by Ub-specific proteases (UBPs), the last glycine amino acid (G) in the c-terminal of ubiquitin was replaced with two

valines (V). After being transformed into *E. Coli* and amplification, mini-prepared plasmid DNA was digested with PstI and SalI, and the fragment was subcloned into a pEGFP-N1 vector. Subsequently, the cDNA for procaspase 3 without the stop codon was PCR amplified and then inserted upstream of the ubiquitin N-degron fusion gene, separated by a short sequence that encodes for the second DEVD peptide. cDNA for human procaspase3 was obtained from GeneCopoeia Inc. (Rockville, MD). By using it as a template, the whole open reading frame minus the last stop codon was PCR amplified by primer pairs (forward 5'-CCGCTCGAGATGGAGAACAACACTGAAAACCTCAGT-3' which has an overhang with a designed XhoI site; backward 5'-TTCTGCAGATTGTGATAAAAATAGAGTTCTTTTGT-3') which has an overhang with a designed PstI site. The PCR reaction was carried out under the following conditions: first denaturation of 3 min at 94°C; 3 cycles of 94°C (10 sec); 65°C (1 min) and 72°C (2 min 30 sec) followed by 30 two-steps cycles 94°C (10 sec) and 68 °C (2min 30 sec). The amplified PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and digested with XhoI and PstI. Digested fragments were purified and inserted upstream of the previously constructed vector. To facilitate screening and stable cell transfection, the whole open reading frame of the chimerical protein was excised from this vector by NheI and NotI, and subcloned into F721 vector^[10] (obtained from Dr. Steve Brandt's lab in the Vanderbilt University Medical Center) by digested with the same pair of enzymes. F721 has a strong elongation factor alpha promoter and a puromycin selection gene linked by an internal ribosome entry site (IRES) sequence. For controls, reporter alone (EGFP and mCherry) cDNA was also removed from their original vectors and subcloned into F721 vector. Right orientation and in-frame ligation in all key junctions were confirmed by direct DNA sequencing. The final vector was designated as F721-EGFP-SA3 (or F721-MC-SA3 when mCherry was used). Vectors to express EGFP or mCherry without conjugated sensor were designated as F721-EGFP and F721-MC.

Cell transfection

The final constructed plasmids were amplified in *E. Coli* DH5⁺ and purified using a Bio-Rad plasmid isolation kit, and transfected into C6, DLD1 or SW620 cells using Lipofectamine 2000 reagent (Invitrogen, CA). The cells were plated at about 70–80% confluence in regular growth media (DMEM + 10% FBS) in 60mm dishes. On day 2, plasmid DNAs were mixed with Lipofectamine and added dropwise to the cells in serum-free Opti-MEM medium. 6 hrs after addition of DNA Lipofectamine mixture, the medium was replaced with regular growth medium; 48 hours after transfection, puromycin were added to a final concentration of 1µg/ml, and cells were grown in this antibiotics medium until all un-transfected cells were killed (3 to 5 days). After expansion, cells were maintained with normal medium supplemented with 0.25µg/ml puromycin. For subsequent experiments using these cells as starting materials, puromycin was omitted from medium.

SDS PAGE and Western blot

Cells were collected by trypsin/EDTA treatment and washed with PBS, and lysed on ice for 15 minutes with M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific, Waltham, MA), supplemented with a cocktail of protease inhibitors (Sigma, St. Louis, MO) at a concentration of 10⁷ cells/ml. Nuclei and insoluble debris were cleared by centrifugation at maximum spin with a microcentrifuge for 30 minutes. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay Kit, Bio-Rad). 30µg of total cell lysate proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were then pre-blocked with 5% non-fat milk in TBST and then incubated overnight at 4 °C with rabbit anti-GFP or anti-caspase 3 antibodies (Santa Cruz biotechnology, Inc., Santa Cruz, CA) diluted with 1% BSA in TBST. After washing three times with TBST, the corresponding HRP-conjugated secondary

antibodies (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) were added and incubated for 1hr at room temperature. The protein bands were visualized by enhanced chemiluminescence (Pierce, Rockford, IL). As a loading control, the same membranes were also blotted with α -tubulin antibody (Santa Cruz).

Fluorescence microscopy of live cells

EGFP or Sensor-EGFP transfected cells (1.0×10^4) were plated in the wells of 8-well chambers (Nalge Nunc International, Rochester, NY) in full growth medium overnight. The cells were directly examined under a fluorescence microscope with cells covered by a thin layer of PBS. Confocal microscopy of the same cells was performed using a Zeiss Inverted LSM510. Images were acquired with appropriate excitation and emission for both GFP and mCherry expressing cells. Transmitted light differential interference contrast (DIC) images were also acquired.

FACS analysis

1×10^6 each of control C6 cells, EGFP or Sensor-EGFP stably transfected C6 cells were trypsinized to single cell suspensions. After washing with PBS, cells were resuspended with 500 μ l of 1% BSA in PBS, and then analyzed on flow cytometer FACSCalibur (BD Biosciences, Mountain View, CA) with multiple lasers. A minimum of 10,000 cells were analyzed per sample, and the fluorescent strength was plotted vs. size scattering.

Effects of drugs on fluorescence signals

Cycloheximide and ZnCl_2 were purchased from Sigma. Bortezomib was purchased from Selleck Chemicals (Houston, TX); PBR06 was synthesized as described previously.^[16] PRB06, Bortezomib and cycloheximide were prepared with DMSO in high concentration stock. ZnCl_2 was dissolved in PBS. 4×10^3 C6 cells (3 groups, non-transfected C6 cells, EGFP or sensor-EGFP expressing C6 cells) were seeded into each well of a black clear bottom 96-well cell culture plate. 24 hours later, medium was removed and replaced with fresh medium or medium premixed with various types of chemicals compounds. After an additional 2 days' treatment, all the media were dumped; the fluorescence signal was measured from plate top and bottom by a Biotek synergy-2 plate reader (BioTek, Winooski, VT).

To examine the change of fluorescent signals at different time points with or without the treatments of various compounds, time course studies were performed. Reporter-expressing C6 cells (1×10^3) were plated in multiple wells of five 96-well plates; along with controls, selective chemical compounds (20 μ M PBR06, 2 μ g/ml Cycloheximide; 100 μ M ZnCl_2) were added into corresponding wells in all the plates after 24 hours of seeding. In day 1, 2, 3, and 5, one plate was withdrawal and used for the determination of fluorescence signal strength directly by plate reader. To compare the results obtained by commercial kits, the similar setups with C6 cells and treatments were analysed with WST-1 Cell Proliferation Reagent (an assay kit based on spectrophotometric detection of soluble tetrazolium salts was purchased from Clontech) to quantify cell growth and viability at the same selected time points. Another pair of comparison is C6 and EGFP-expressing C6 cells, the correlation of fluorescent signal vs proliferation were determined. Cell proliferation were determined with CyQuant cell proliferation assay kit (Invitrogen). The setups were similar to those used for time course omit compound treatment. At each selected time point started at 24 hours, a pair of plates was retrieved, the plate with EGFP-expressing cells is used for direct fluorescent reading, and the plate with control C6 cells is used for total DNA quantitation by following manufacturer's protocol. For some setups with compound treatments, apoptosis assay based on Caspase 3/7 activity was also performed (caspase-Glo kit, Promega, Madison, WI).

High throughput screening of anticancer compounds

C6 and DLD1 cells transfected with the reporter system were setup in 386-well plates for concurrent and subtractive drug selection. The appropriate number of cells that will yield ~100% confluence after 3 days was determined individually (1000 cells for C6 and 500 cells for DLD1). Cells were plated in 30 μ l of complete medium in 384-well plates and allowed to settle overnight. Twenty-four hours after seeding, compounds from the NIH Clinical Collection (NCC) library was prepared with each compound at a stock concentration of 120 μ M in DMSO, and then diluted to 40 μ M by same growth media. 10 μ l of each chemical compound was transferred into the cell plates to yield the final screening concentration of 10 μ M by using a high-precision multi-well pipetting instrument (Bravos, Velocity 11). No DMSO and no drug (DMSO alone) controls were setup against each of the cell lines in the first and last two columns. The positive hits were confirmed by simply repeat the screening at a single concentration. When IC₅₀ was determined, the selected compounds were serially diluted 1:3 with complete medium by 9 times (for a concentration range from 30 μ M to 1.5nM); 10 μ l of this dilution was added to each well containing 30 μ l of medium and cells. After 2 days, the cell media was dumped, fluorescence was measured per well from the top by a plate reader.

Optical imaging

To image tumor size and size change, 1 $\times$ 10⁶ SW620 cells stably transfected with mCherry as reporter were injected subcutaneously into hind limb of athymic nude mice. After two weeks, planar fluorescence imaging was performed using an IVIS 200 CCD camera (Caliper, Hopkinton, MA). Anesthesia was induced and maintained with isoflurane (1.5% in 98.5% O₂). Fluorescence imaging was performed using excitation and emission filters tuned to the wavelength of DS red (mCherry). For tumor metastasis tracking, 1 $\times$ 10⁶ mCherry reporter transfected SW620 cells were injected into athymic nude mice through tail vein. All experimental mice were fed with a special formulated diet to reduce background fluorescence. Four weeks later, fluorescence tomography was performed using an FMT 2500 imaging system (PerkinElmer, Waltham, MA). Animals were placed in an imaging cassette under anesthesia. A diode laser was scanned over the abdomen of the animal and fluorescence emission was used to reconstruct the fluorescence distribution.

Animal experiments

Female athymic nude mice were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). All procedures involving animals were approved by our Institutional Animal Care and Usage Committee at Vanderbilt University (protocol number M/09/072), and in fully compliance with United states Animal Welfare Act and Regulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

A Reporter System for Imaging and HTS

Figure 1.

Schematic illustration of a novel reporter system. A) The plasmid map of the construct shows major components and their relative position. B) Chimeric polypeptides consisting of a switch sensor fused to a reporter molecule. A caspase 3 cleavage site (DEVD) was inserted in two different locations. Apoptosis (activation of caspase 3) will lead to cleavage and produce two unstable c-terminal portions of proteins. The released N-terminal procaspase 3 will be further processed to convert to active caspase 3. This feedback loop will further amplify the signal.

Figure 2.

Characterization of the expressed reporter proteins. A) Confirmation of fusion protein expression. C6 cells were stably transfected with EGFP (lane 1) or Sensor-EGFP (lane 2) vectors. 30μg of whole cell lysate were separated by SDS-PAGE and blotted with antibody against caspase 3 (left panel) or against GFP (right panel). Both show a fusion protein with MW around 75kd and some smaller products. B) Nongated fluorescent activated cell sorting demonstrated more than 99% of drug (puromycin) resistant cells are transgene positive. Left panel is untransfected control cells. Middle panel shows cells transfected with sensor-EGFP fusion, which are less bright, and show a wider range of fluorescence signal compared to those cells transfected by EGFP alone (right panel). C) Confocal microscopy reveals the localization of EGFP and Sensor-EGFP fusion in C6 cells. While EGFP distributes throughout cells (left panel), the sensor-EGFP fusion is excluded from the nuclei, and shows a more distinctive granular distribution (right panel). Images were taken at 40x amplification.

Figure 3.

Drugs capable of inducing apoptosis reduce the overall fluorescent signals of the reporter system. A) 10 μM Camptothecin (CPT) was added into C6 cells expressing the reporter system. After 3 days of treatment, the cells were observed under a fluorescence microscope (20x amplification). Both cell numbers and fluorescence signals were notably decreased in the reporter expressing C6 cells. B) Measurements of fluorescent intensity of cells expressing the reporter system after being treated with different compounds. EGFP- or reporter-expressing C6 cells (4×10^3) were plated in each well of black 96-well plates; four different chemical compounds (PRB06, Cycloheximide, Bortezomib and ZnCl₂) were added into corresponding wells. 2 days later, the fluorescence signal strength was determined by plate reader. Each data point was the average from 6 wells, standard deviation are indicated as error bars. Compare to control, the p value for TSPO and cycloheximide and Bortezomib treatment is less than 0.003, for ZnCl₂ treatment the p value is about 0.009. C) Time course of reporter signals for selected chemical treatments. C6 and reporter-expressing C6 cells (1×10^3) were plated in multiple wells of five 96-well plates for each group; selective chemical compounds were added into corresponding wells in all the plates after 24 hours of seeding (5 plates in each group were setup identically), the compound concentrations used in these experiments are the same as indicated in Panel B. In day 1, 2, 3, and 5 after treatments, one plate from each group were retrieved from incubator, the plate with reporter-expressing cells was used for the determination of fluorescence signal strength by plate reader; D) The C6 cell plates were analysed with commercial colorimetric assay kit (WST-1 Cell Proliferation Reagent, Clontech) to quantify cell growth and viability. WST-1 assays were performed with 45 minute incubation time and absorbance was recorded at 440nm. Each data point was the average from 6 wells; standard deviations were shown as error bars.

Figure 4.

This reporter system can be used in high through put screening. A) Reproducibility and robustness of the assay, the variances of C6 and DLD1 cell lines treated with or without 10μM camptothecin (CPT) in a 384-well plate were determined by sampling data from 64 wells for each group. The calculated z factor was 0.59 and 0.61 for C6 and DLD1 cells respectively. B) Scatter diagram of hits discovered for glioma C6 cells by screening NIH Clinical Collection (NCC) library. Two 384-well plates were seeded with 1000 cells in 30μl of complete medium. Twenty-four hours later, the chemical compounds from the NCC library were prepared at 40μM. 10μl of each chemical compound was transferred into the cell plates to yield the final screening concentration of 10μM by using a high-precision multi-well pipetting instrument (Bravos, Velocity 11). No DMSO and no drug (DMSO alone) controls were setup in the first and last two columns. After 2 days of treatment, cell medium was removed, and the florescent strength was recorded by plate reader. The reading on and under horizontal line (at or more than 6 standard deviation) were counted as hits.

Figure 5.

Cell morphology and dose response curve for selective hits. Cell morphology and cellular structure imaged after HTS for C6 (A) and DLD1 cells (B). The plate after reading was examined under a fluorescent microscopy. The images at 20x amplification were taken from the wells of control (no added compound, DMSO only), Docetaxel, Homoharringtonine, and Itavastatin as labeled. C6 cells (A); DLD1 cells (B); (C) Dose response curve determined by using the reporter system for C6 cells treated with Etoposide at indicated concentration for 48 hours. Each data point represents the average of a triplicate reading and the standard deviations plotted as error bars, data fit was carried out by GraphPad.

Table I

Hits showing inhibitory effects on C6 and DLD1 cells by screening with the NCC library

Name	C6 Cells	DLD1 Cells	Comment
Docetaxel	****	****	Anti-mitotic chemotherapy
Methyltestosterone	***	***	Steroid, treat breast cancer
Flubendazole	****	***	Antilekemia
Topotecan	****	****	Topoisomerase I inhibitor
Doxorubicin	****	****	Anthracycline antibiotic
Vinorelbine bitartrate	****	****	Alkaloids
Carmofur	****	**	Antineoplastic agent.
Diphenylcyclopropenone	****	****	Hair loss & Bald
Artesunate	****	**	Induce apoptosis
Itraconazole	****	**	Antifungal
Cytarabine	*****	***	Interfering with DNA synthesis
Triptolide	*****	****	Antitumors
Trileptal	****	*	Treat brain tumor
Idarubicin	****	****	Topoisomerase II inhibitor
Piribedil	**	*	Antiparkinsonian
Nisoldipine	****	***	Calcium channel blocker
Epirubicin	****	****	Anthracycline cancer drug
Vindesine sulfate	****	****	Alkaloid
Imatinib mesylate	**	**	Kinase inhibitor
Irinotecan	*****	****	Topoisomerase I inhibitor
6-azauridine	**	***	Antineoplastic
Homoharringtonine	*****	*****	Treat CML AML
Raltitrexed	*****	****	Antimetabolite
Resveratrol	*	***	Phenol, anticancer
Etoposide	****	*	Topoisomerase II inhibitor
Mevastatin	*****	*	Statins
Dactinomycin	*****	*****	Inhibit transcription
Itavastatin	*****	****	Statins
Bortezomib	*****	*****	Proteasome inhibitor

Degree of inhibition 5* > 75%; 4* between 60 to 75%; 3* between 45 to 60%; 2* between 30 to 45%; 1* < 30%