**High-throughput screening for Survivin and Borealin interaction inhibitors in
hepatocellular carcinoma**

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Abstract

Survivin, a key member of the chromatin passenger complex (CPC), is often highly expressed in human cancers, making it a promising target for cancer treatment. Out of the numerous reported Survivin inhibitors, YM155 is only one entering clinical trial, but was recently failed in the Phase II trial. It is important to develop Survivin inhibitors with new strategies. We recently reported that both Survivin and its binding protein Borealin in the CPC complex are essential for the development of hepatocellular carcinoma, suggesting that disrupting the interaction between Survivin and Borealin would be a promising strategy. Here, we developed a high-throughput screening based on bimolecular fluorescence complementation (BiFC) technology in cultured cells, which allowed the identification of small chemical inhibitors specifically blocking the Survivin and Borealin interaction. Primary hits from BiFC were further validated in an *in vitro* AlphaScreen system, which detects the direct interactions of Survivin and Borealin. Etoposide was identified as one of the effective hits. Direct interaction between Survivin and Etoposide was confirmed by surface plasmon resonance assay, and molecular docking analysis suggested the structural information on how Etoposide inhibits the Survivin and Borealin interaction. These results demonstrate a screening system to identify small molecule chemicals inhibiting Survivin and Borealin interaction. In future, an even larger scale screening may lead to identification of better Survivin and Borealin inhibitors.

Key words

Survivin; Borealin; PPI inhibitor; BiFC; HCC; Etoposide

Introduction

Survivin (BIRC5) is the smallest member of the inhibitor of apoptosis protein (IAP) family, bearing a single baculovirus IAP repeat (BIR) domain. It is highly expressed in most human cancers, but not in normal, terminally differentiated adult tissues [1]. Previous studies have shown that Survivin promotes the tumor progression by multiple pathways such as inhibition of apoptosis [1], dysregulation of cell proliferation [2] and facilitation of angiogenesis [3]. The overexpression of Survivin is associated with tumor malignancy, chemotherapy resistance and local tumor recurrence [4, 5], making it an attractive target for human cancer treatment.

Various approaches have been developed to impair the function of Survivin. YM-155 was identified in high-throughput screening for small molecule inhibitors of *Survivin* transcription using a *Survivin* promoter-luciferase assay. YM-155 showed potential anti-cancer activity in preclinical models and was the only one entering the clinical trial [6]. However, YM-155 was failed in the phase II clinical trial, largely due to its toxicity effect on healthy tissues. The toxicity and side effects of YM-155 is likely caused by its non-specificity to target other pathways besides Survivin [7, 8]. Such non-specificity is often reported for inhibitors identified using transcription reporter assays [9]. Phosphorothioate antisense oligonucleotides were designed to suppress Survivin mRNA and protein expression. LY2181308 is such an oligonucleotide that produces strong anti-cancer activity in preclinical models [10]. LY2181308 had been also used in several clinical trials while no exciting results have been reported [11]. Other strategies based on the disruption of the interactions between Survivin and Hsp90 [12] or between Survivin

and Smac/DIABLO [13] was developed. However, the biological functions between Survivin and these proteins were unclear in tumorigenesis, and no promising results regarding the application of these compounds were so far reported.

Hepatocellular carcinoma (HCC) is the one of the most common malignancies and common cause of cancer-related mortality worldwide. Sorafenib is the only approved targeted drug for advanced HCC. It is urgently needed to develop new drugs for this dreadful disease. Our previous data showed that Survivin plays a major role in regulating cell survival during the initiation of HCCs [14]. In a diethylnitrosamine (DEN) induced HCC model, we observed reduced HCC formation in hepatocyte-specific Survivin knockdown mice. Besides the DEN-induced HCCs, we found that lack of Survivin also showed remarkable HCC regression in multiple mouse models [15], providing compelling genetic evidence that targeting Survivin is an effective therapeutic strategy for HCCs. In consistence with the essential functions of Survivin in hepatocyte proliferation, we found that Borealin, another component of the CPC complex, was highly expressed in human HCCs. Hepatocyte-specific Borealin deficiency suppressed Δ N90- β -Catenin/c-Met-induced HCC development in the similar way to Survivin deletion [16]. These data suggest that the CPC complex is required for liver cancer development and targeting Survivin and Borealin would be efficient to eliminate HCC cells. Notably, direct interaction between Survivin and Borealin has been demonstrated by the crystalized Survivin-Borealin complex with N-terminal peptide of INCENP [17]. The crystal structure analyses revealed that the hydrophobic pocket of Survivin formed by Leu6^{Sur}, Trp10^{Sur}, Phe93^{Sur}, Phe101^{Sur}, and Leu102^{Sur} recognized the apolar residues of Borealin, including Leu61^{Bor}, Leu65^{Bor}, Trp70^{Bor}, Leu71^{Bor}, Tyr73^{Bor}, and Phe74^{Bor}.

These structure data strongly indicate that Survivin and Borealin may function in concordance to promote HCC development. It is noteworthy that hepatocytes deficient in either Survivin or Borealin did not show apparent adverse effects on adult liver functions, indicating that direct targeting the Survivin and Borealin interaction may be safe for HCC patients, who usually display impaired liver functions.

Here we developed a high-throughput screen method based on the principle of the bimolecular fluorescence complementation (BiFC) [18] to discover novel Survivin and Borealin interaction inhibitors. AlphaScreen assay was further applied to confirm the hits identified by BiFC. Etoposide was thus discovered to show potent activity in disrupting Survivin and Borealin interaction *in vitro* and anti-tumor activity *in vivo*.

Materials and methods

Reagents

Primary rabbit anti-GFP antibody (Genetex, GTX113617), mouse anti- α -Tubulin (Sigma-Aldrich, T9026), HRP Goat Anti-Rabbit IgG Antibody (Peroxidase) (Vector Laboratories, PI-1000), HRP Horse Anti-Mouse IgG Antibody (Peroxidase) (Vector Laboratories, PI-2000), all were 1:1000 diluted for use.

Plasmid construction

We cloned the N-terminal 1-157 amino acids of a yellow-shifted variant of the green fluorescent protein of *Aequorea victoria* (Venus) into the mammalian expression vector pcDNA3.1/Hygro (-) (Invitrogen), and the C-terminal 158-237 amino acids of Venus into pcDNA3.1/myc-His (-) A (Invitrogen). The two vectors were named as NV and CV respectively. Survivin was inserted into the C-terminus of NV, named as Sur-NV. Borealin was inserted into the N-terminus of CV, named as Bor-CV.

Cell culture and transfection

The human cell line HEK293T, the liver cancer cell line Tong and HLE all were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone) and 0.5 mg/ml penicillin-streptomycin (Life Technologies). Another liver cancer cell line Snu423 was cultured in GIBCO RPMI Media 1640 (Thermo Fisher Scientific) medium. HEK293T cells were transfected using FuGENE HD (Promega) at a 1:3 DNA-to-reagent ratio with 60 ng DNA and 180 nl transfection reagent per well of a 384-well plate.

BiFC screening assay

BiFC assays were conducted in 384-well plates (Corning, 3712). Cells were transfected as a single-batch, and then 8,000 cells per well were seeded in 30 μ l of medium using Multidrop Combi dispenser (Thermo Fisher Scientific). 4 hours after transfection, 30 μ l of 20 μ M compound solutions were transferred to assay plates using Bravo liquid handling machine (Agilent). 48 hours after compound treatment, for assay optimization, fluorescence images were taken on Operetta (PerkinElmer). Normalized mean fluorescence intensity were quantified using Harmony software (PerkinElmer). For large scale screening, phase contrast and fluorescence images were acquired on the IncuCyte FLR (Essen BioScience), and quantified using software IncuCyte 2011A (Essen BioScience). The fluorescence intensity per mm^2 was divided by cell confluence to get the normalized mean fluorescence intensity per well.

AlphaScreen assay

Survivin protein was used at 250 nM, and Borealin protein was 100 nM. All reactions were performed in 1 $\times$ PBS buffer (pH 7.4, with 1 mg/ml BSA) in OptiPlate-384 (PerkinElmer). Pairs of recombinant proteins and compounds were incubated together for 1 h at 25 $^{\circ}$ C. Then, Nickel Chelate Acceptor beads and Streptavidin Donor beads were added to a final concentration of 0.02 mg/ml and incubated for another 1 h at 25 $^{\circ}$ C in sequence. The signals generated by binding interactions were measured using EnVision (PerkinElmer). A Biotin-His₆ linker peptide (PerkinElmer) was used for counter screen.

SPR assay

Measurements were performed on an Octet instrument (ForteBio). Biotinylated Borealin and His-tagged Survivin were immobilized on SA or NTA sensors separately. For the loading step, protein concentration was 20 µg/ml. Compound concentration for the association and dissociation steps was 10 µM.

Real-time quantitative polymerase chain reaction

Total RNA was prepared from liver cancer cell lines using Trizol (Invitrogen). Reverse transcriptase PCR was performed using M-MLV reverse transcriptase (Promega). Real-time PCR reactions were performed with SYBR Premix Ex Taq (Takara) and the 7500 Fast Real-Time PCR Systems (Applied Biosystems). All runs were accompanied by the β -actin internal control.

Cell viability assay

Cells were seeded in 384-well plates and the optimal cell number for every HCC cell line was determined to ensure that each one was in growth phase at the end of the experiment. Compounds were serially diluted to 8 concentrations generating a dose response range 50 µM to 0.39 µM. Cell viability was measured after 72 h of drug exposure using CellTiter-Glo reagents (Promega) and detected by EnVision.

Protein-compound docking

The binding conformation of Etoposide to the X-ray structure of Survivin/Borealin complex (PDB ID: 2QFA) is investigated by Induced Fit Docking of Schrodinger suite.

Statistical analyses

A standard two-tailed Student's *t*-test was used for some experiments. *P*-values below 0.05 (*) or 0.01 (**) were considered as statistically significant or highly significant, respectively. Calculate the *Z'* factor for the screening assay using the formula: $Z' = 1 - (3(SD_{\max}) + 3(SD_{\min}) / \text{Mean}_{\max} - \text{Mean}_{\min})$. The coefficient of variation is defined as the ratio of the standard deviation to the mean, and expressed as a percentage: %CV.

Results

Development of a high-throughput screen system based on bimolecular fluorescence complementation (BiFC) technology in HEK293T cell

BiFC enables direct visualization of protein-protein interactions in live cells [19]. This approach relies on a protein-protein interaction, specially Survivin and Borealin interaction in this study, to juxtapose the two non-fluorescent, complementary fragments of Venus, thus promoting complementation of the functional Venus structure [20]. An important requirement for this assay is the expression of both complementary fragments in the same cell simultaneously. The BiFC screening assay involves co-transfection of two plasmids, so as to express the target proteins as fusions with the respective N- and C-terminal Venus fragments (designated as NV and CV, respectively). The schematic diagram of the assay used in this study was shown in Fig. 1A.

To characterize the BiFC screen system, HEK293T cells were transiently co-transfected with different pairs of plasmids, followed by fluorescence microscope detection. The cells co-transfected with the Survivin-NV (Sur-NV) and Borealin-CV (Bor-CV) plasmids produced bright green fluorescence with apparent localization in the nuclei (Fig. 1B), which validated functional Survivin and Borealin expression and interaction. In contrast, as negative controls, the cells co-transfected with NV and CV, or Sur-NV and CV, or Bor-CV and NV exhibited merely weak background fluorescence signals (Fig. 1B). The fluorescence signals in these controls were significantly lower than the signals from Sur-NV and Bor-CV cells (Fig. 1C). Furthermore, we constructed a Survivin mutant at L98A, F101A, L102A and a Borealin mutant at W70E, F74E based on their binding structure knowledge [8]. The combinations of wild-type Sur-NV and mutant Bor-CV

plasmids, or *vice versa*, only showed weak background fluorescence signals as negative controls in HEK293T cells (data not shown). The successful expressions of recombination proteins were all validated by Western blot analysis (Fig. 1D). These data indicated that the functional specific interaction of Survivin and Borealin could be detected and analyzed with image-based fluorescence intensity at the cellular level by BiFC approach.

Large scale primary screening for small chemical inhibitors specifically blocking the Survivin and Borealin interaction by BiFC Assay

For high-throughput screening using live cells, it is necessary to optimize the assay to 384-well plate format. To that purpose, a robust and simple cell transfection method suitable for 384-well plate manipulation is required for our BiFC screening assay. We compared the transfection effects in adherent HEK293T cells and suspended HEK293T cells. For adherent cells, we dispensed the transfection complexes onto pre-seeded and spread adherent cells in a 384-well plate. Whereas, for suspended transfection, the detached cells were mixed with transfection complexes and immediately seeded into a 384-well plate. We found that either method was able to produce distinct BiFC fluorescence, but the signal from suspended method was much higher at 40 hours after transfection (Fig. 2A). Moreover, considering of the efficiency of one-step dispensing, the suspended transfection was chosen for this screening.

For the primary BiFC screening, we screened a compound library composing 10400 small molecule compounds with diverse structures and compounds separated from natural herbal plants. A cell bank of defined passage was established and cells were

propagated for no more than 10 passages in culture throughout the screening. Suspension HEK293T cells were transiently co-transfected with Sur-NV and Bor-CV during dispensing into 384-well assay plates. Four hours after transfection, the chemical compounds were transferred into the assay plates with a final concentration at 10 μ M using Bravo liquid handling workstation. The cells were cultured in an incubator for 48 hours before image detection by IncuCyte FLR live cells imaging system. Each assay plate contained several positive controls (Sur-NV and Bor-CV) and several negative controls (NV and CV), in the presence of compound vehicle control (0.1% DMSO). The Z' factor for most assay plates from the primary screen were larger than 0.5 (Fig. 2C), and the coefficient of variation (%CV) of most assay plates were less than 10% (Fig. 2D), indicating a robust assay performance of this screening. The normalized fluorescence inhibition rate of each compound was calculated relative to the DMSO-treated controls. Totally 146 primary hits with > 50% inhibition rate for Survivin and Borealin interaction were selected out (Fig. 2B).

Primary hits validation in an in vitro secondary screening system adopting AlphaScreen technology

For drug screening, no matter phenotypic or target-based screening, it is vital to have combined evaluating assays to cross validate the hits, better in a different assay. We tried a biochemical protein-protein interaction assay adopting AlphaScreen technology to further validate the primary hits. We produced recombinant His-tagged Survivin wild-type and Survivin mutant (L98A, F101A, L102A) proteins, and GST-tagged Borealin wild-type and Borealin mutant (W70E, F74E) proteins which were biotinylated

for use. These two mutants would be incapable of binding to wild-type Survivin or Borealin respectively.

As expected, in our AlphaScreen system, wild-type Survivin and Borealin proteins mixture produced stronger signal compared to buffer control (Buffer and Bor) (Fig. 3A), indicating successful direct binding of these two proteins. In contrast, as negative controls, protein mixtures of Survivin and Borealin mutants, or Survivin mutant and Borealin, or Survivin mutant and Borealin mutant, all produced significantly lower signals than that produced in wild-type Survivin and Borealin (Fig. 3A), further confirming the specificity of this assay system. Next, the 146 primary hits were evaluated using the AlphaScreen assay. 13 hits with > 30% inhibition rate were selected out (Fig. 3B). A counter screen for these 13 hits was performed using a Biotin-His₆ linker peptide to identify false positive chemicals, and 2 false positive hits were removed (data not shown). Out of the final 11 hits, one representative hit with known bioactivities was the anticancer drug Etoposide (Fig. 3D), which efficiently disrupted Survivin and Borealin interaction in our BiFC and AlphaScreen assay (Fig. 3C). Other hits were under further structure-activity relationship study and development.

Etoposide bound to Survivin directly to interfere Survivin and Borealin interaction and specifically inhibited the proliferation of HCC cells with high Survivin expression.

In order to investigate the detailed molecular mechanism by which Etoposide interacted with Survivin and Borealin, we performed molecular docking first. Crystal structure of the regulatory core of the CPC was previously determined with the resolution of 1.4 Å (PDB ID: 2QFA) [17]. The structure data showed that residues Trp70 and Leu71 of

Borealin dock into the hydrophobic pocket presenting on the BIR domain of Survivin with residues Leu6, Trp10, Phe93, Phe101 and Leu102 (Fig. 4A). Calculation of electrostatic surface charge revealed a highly acidic (red) and basic patch (blue) on the molecular surface of Survivin. Etoposide located in the same hydrophobic pocket of Survivin while forming two hydrogen-bonds with Asp16 of Survivin, occupying the Borealin residues. These data suggested that Etoposide likely blocked the Survivin and Borealin through this interaction surface (Fig. 4A). Moreover, the surface plasmon resonance assay showed direct binding of Etoposide to recombinant Survivin protein, but not with Borealin, which was consistent with the docking result (Fig. 4B). These data together suggested that Etoposide directly bound to Survivin so as to competitively block the binding of Borealin.

The anti-tumor activity of Etoposide was verified in liver cancer cell lines. Quantitative PCR results from three tested HCC cell lines displayed that the mRNA level of Survivin was high in Tong and HLE, but much lower in Snu423 (Fig. 4C). CellTiter-Glo luminescent cell viability assay results showed that Etoposide specifically inhibited the proliferation of Tong and HLE in a dose-dependent manner, while little cytotoxic effect was observed in Snu423 (Fig. 4D), indicating that Etoposide imposed better efficacy on Survivin-dependent HCC.

Discussion

Our previous data had shown that hepatocyte-specified Survivin or Borealin deficiency resulted in the HCC regression following hepatocyte mitotic arrest, hypertrophy, and genome polyploidy, indicating a functional concordance of Survivin and Borealin in promoting HCC development. Borealin binds directly to Survivin therefore enhancing the localization and function of the CPC complex during mitosis. Disruption of the interaction between Survivin and Borealin appeared to be a promising strategy to inhibit HCC growth. In this report, we established a cellular level high-throughput screening system in 384-well plates to discover Survivin-Borealin interaction inhibitors based on the principle of BiFC. BiFC screening assay allows direct visualization of protein-protein interactions (PPIs) in their normal cellular environment. Using Venus fluorescent protein as the indicator of PPI, the experiment procedure is relatively simple and economic without complicated washing processes or reaction substrates manipulations. Its stable signal-to-background ratio guarantees the high quality of large scale screening data. The BiFC screening assay turns out to be a powerful and convenient approach for discovering PPI inhibitors in live cells.

By performing the BiFC assay, we screened 10,400 compounds, and then got 11 validated hits. Etoposide, was used as a representative hit to verify the concept of inhibiting HCC cells growth through blocking Survivin and Borealin interaction. The computational docking result showed that Etoposide protruded into the same hydrophobic pocket of Survivin which recognizes the apolar residues of Borealin (Trp70, Leu71), thus blocking the direct interaction between Survivin and Borealin. Also, the direct interaction between Survivin and Etoposide was detected by means of SPR assay in the experiment

level. The results from Etoposide clearly supported the effectiveness of this molecular-targeted drug screening system, and implying a precise therapeutic use potential of Etoposide on Survivin-dependent HCC or even other cancer types.

It would be interesting to characterize other hits because they were novel in anti-cancer or in structure. Detailed structure-activity relationship studies and functional activities would be evaluated systematically in biochemical level, cellular level and animal models in following research. Also, larger scale screening would be carried out for discovering more promising hits on this target. Altogether, our findings suggest that Survivin and Borealin interaction could be a valuable target for drug screening to treat HCC.

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Figure Legends

Fig. 1. The BiFC assay for screening Survivin and Borealin interaction inhibitors was established. (A) The schematic diagram of the principle of this BiFC screening assay. (B-D) HEK293T cells were co-transfected with NV & CV, NV & Bor-CV, Sur-NV & CV, Sur-NV & Bor-CV, respectively. (B) The fluorescent Venus was detected only in Sur-NV & Bor-CV transfected cells and located in nuclei. Nuclei were identified by DAPI. Scale bar: 50 μ m. Results are representatives of three independent experiments. (C) Normalized mean fluorescence intensity of each transfection condition were analyzed using 3 image fields with more than 1,000 cells. ** $P < 0.01$ compared to Sur-NV & Bor-CV. (D) The expression of transfected proteins were detected by Western blot.

Fig. 2. Large scale BiFC primary screening in cultured cells. (A) The time curves of Venus fluorescence in two different transfection methods. Suspension transfection was selected for the whole screening. (B) Overview of the primary screening data. Each spot represents a compound at 10 μ M. Data are relative to the DMSO-treated control. Compounds with over 50% inhibition effects were selected out as primary hits. The Z' factor (C) and %CV (D) for all screened assay plates were presented. The average Z' factor was greater than 0.5, and the average %CV is less than 10%.

Fig. 3. Hits validation in a biochemical secondary screening assay. (A) The interaction between Survivin and Borealin was detected by AlphaScreen assay. Survivin mutant and Borealin mutant proteins served as negative binding controls. ** $P < 0.01$ compared to Sur & Bor. (B) Overview of the AlphaScreen data for the primary hits. (C) Inhibitory effect of Etoposide, one representative confirmed hit, on Survivin and Borealin

interaction in the BiFC and the AlphaScreen assay compared to DMSO-treated controls.

* $P < 0.05$, ** $P < 0.01$. (D) Chemical structure of Etoposide. MW: molecular weight.

Fig. 4. Etoposide was proved to be a potent functional inhibitor for Survivin and

Borealin interaction. (A) Docking conformation of Etoposide to Survivin. Etoposide

binds to Survivin in the hydrophobic pocket, that previously occupied by Trp70 and

Leu71 of Borealin. Molecular surface of Survivin is shown with calculation of

electrostatic surface charge (red: negative charge; blue: positive charge). Etoposide is

shown in balls and sticks with carbon atoms in green, oxygen atoms in red, and hydrogen

atoms in white. Borealin is presented as cartoon in cyan with Trp70 and Leu71 as sticks.

(B) KD value were evaluated using SPR assay. Etoposide directly bound to Survivin with

a KD at $3.52 \pm 0.76 \mu\text{M}$, but didn't bind to Borealin. (C) RT-PCR results from three HCC

cell lines showed that the mRNA level of Survivin was high in Tong and HLE, but much

lower in Snu423. (D) Etoposide specifically inhibited the proliferation of Tong and HLE

in a dose-dependent manner, while showed little cytotoxic effect on Snu423 ($n=4$). Data

are means $\pm$ SD relative to DMSO-treated controls.

Figure1

Figure2

Figure3

Figure4