Discovery of inner centromere protein-derived small peptides for cancer imaging and treatment targeting survivin

Takeshi Fuchigami¹ | Natsumi Ishikawa¹ | Iori Nozaki¹ | Yusuke Miyanari² | Sakura Yoshida¹ | Motohiro Yamauchi³ | Ayumi Soejima¹ | Mamoru Haratake⁴ | Morio Nakayama¹

¹Department of Hygienic Chemistry, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
²Okazaki Institute for Integrative Bioscience, Okazaki, Japan
³Department of Radiation Biology and Protection, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan
⁴Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan

Correspondence
Takeshi Fuchigami and Morio Nakayama,
Department of Hygienic Chemistry,
Graduate School of Biomedical Sciences,
Nagasaki University, Nagasaki, Japan.
Email: t-fuchi@nagasaki-u.ac.jp (T.F.); nakmorio@gmail.com (M.N.)

Funding information
Grant-in-Aid for JSPS Research Fellow, Grant/Award Number: 17J11488; Grant-in-Aid for Scientific Research (C), Grant/Award Number: 15K09895; Grant-in-Aid for Young Scientists (B), Grant/Award Number: 21791180

Abstract
Survivin belongs to the inhibitor of apoptosis protein family, which is consistently overexpressed in most cancer cells but rarely expressed in normal adult tissues. Therefore, the detection and inhibition of survivin are regarded as attractive strategies for cancer-specific treatment. In this study, we designed and synthesized 7-19 residues of inner centromere protein (INCENP)-derived small peptides (INC peptides) as novel survivin-targeting agents. The INC peptides showed binding affinity for the human survivin protein ($K_d$ = 91.4-255 nmol L$^{-1}$); INC16-22, which contains residues 16-22 of INCENP, showed the highest affinity (91.4 nmol L$^{-1}$). Confocal fluorescence imaging showed consistent colocalization of FITC-INC16-22 and survivin in cell lines. Nona-arginine-linked INC16-22 (r9-INC16-22) rendered INC16-22 cells penetrable and strongly inhibited cell growth of MIA PaCa-2 cells (52% inhibition at 1.0 µmol L$^{-1}$) and MDA-MB-231 cells (60% inhibition at 10 µmol L$^{-1}$) as determined by MTT assays. The exposure of MIA PaCa-2 cells to 40 µmol L$^{-1}$ r9-INC16-22 apparently reduced the intracellular protein expression levels of survivin. However, cleaved caspase-3 was significantly increased in cells treated with r9-INC16-22, even at 10 µmol L$^{-1}$, compared to untreated cells. Flow cytometry revealed that r9-INC16-22 strongly induced apoptosis in MIA PaCa-2 cells. These results indicate that the cytotoxic effects of r9-INC16-22 could be mediated mainly through the disruption of survivin-dependent antiapoptotic functions and partly because of the direct degradation of the survivin protein. Our findings suggest that INC peptides can act as useful scaffolds for novel cancer imaging and anticancer agents.

KEYWORDS
anticancer, apoptosis, cancer imaging, peptide, survivin

1 | INTRODUCTION

Survivin, the smallest protein (16.5 kDa) among the inhibitor of apoptosis protein (IAP) family, is one of the most cancer-specific proteins¹,²; it is highly overexpressed in most cancers, but barely detected in the majority of normal cells.³⁻⁶ Survivin is involved in at least 2 essential cellular processes, inhibition of apoptosis and regulation of the cell cycle.⁵ Furthermore, it participates in another antiapoptotic effect by...
stabilizing X-linked IAP (XIAP), which strongly suppresses caspase-mediated cellular apoptosis, to stabilize XIAP.7 In addition, survivin interacts with the apoptosis-inducing protein Smac/DIABLO to delay its release and to inhibit complex formation with XIAP, resulting in the suppression of caspase-induced apoptosis.8,9 Unlike other IAPs, survivin is known as one of the essential components for cell division regulation.10 It forms the chromosomal passenger complex (CPC) with inner centromere protein (INCENP), Borealin, and Aurora B. Chromosomal passenger complex plays an important role in the regulation of chromosome-microtubule association, proper spindle assembly, and execution of appropriate cytokinesis.2,10,11

The abnormal expression of survivin in cancers is attributed to various factors, including microRNAs, p53 mutants, nuclear factor-κB, receptor tyrosine kinases, and numerous signaling cascades, such as the PI3K/Akt and signal transducer and activator of transcription-3 (STAT3) pathways.12,13 Numerous reports have indicated that overexpression of survivin correlates with malignancy, poor clinical outcome, and recurrence of various cancers.14 Recent studies suggest that survivin is involved in the regulation of cancer stem cells that can increase radiation and drug resistance of cancers.10,15

Therefore, survivin has recently attracted interest as a promising cancer biomarker and a target for diagnostic and therapeutic agents. Unfortunately, there are currently no clinically available diagnostic or therapeutic agents targeting survivin. Several strategies for survivin-targeting anticancer therapy are being developed, including inhibition of survivin expression, destabilization of the survivin protein, and interaction with other oncoproteins.14 YM155 is a small molecule that regulates the survivin gene promoter to inhibit survivin mRNA expression. It has shown significant antiproliferative, antimitotic, and apoptosis induction activities in preclinical investigations.16-18 However, YM155 has failed to show significant anticancer effects in phase II clinical trials of several cancers.19-21 Recent studies have suggested that YM155 anticancer activity is partly due to mechanisms unrelated to survivin expression suppression, including downregulation of Mcl and DNA damage.22,23 LY2181308 is an antisense oligonucleotide targeting survivin mRNA that shows apoptotic effects in cancer cell lines.24,25 However, a phase II clinical trial of this agent has not indicated beneficial anticancer effects in patients with prostate cancer.26 Small molecules targeting the survivin dimerization domain can induce survivin destabilization and degradation. Although several of these small molecules have shown significant anticancer effects through survivin degradation in vitro and in vivo, there have been no reports regarding this type of agent proceeding to clinical studies.27-30 For cancer-specific diagnosis, we have recently reported several radiolabeled small molecules targeting the survivin dimer site as nuclear medicine imaging agents; however, these agents have limitations, such as the lack of in vivo pharmacokinetics and specificity.31,32

Survivin contains a single N-terminal baculovirus IAP repeat domain linked to a long C-terminal α-helix coiled domain.33 In solution, purified survivin exists in a bow tie-shaped dimerization form.33,34 Both dimer and monomer survivin have been implicated in the inhibition of apoptosis, although the detailed dimer-monomer balance regulation has not yet been elucidated.35 However, survivin plays an essential role in CPC as a monomer that binds to INCENP and Borealin at the C-terminal helical coiled-coil domain of survivin.36 Accordingly, we suggest that survivin-binding molecules targeting the C-terminal domain of survivin enable both monomer and dimer states of survivin, which could not intrinsically be achieved by survivin dimer-targeting agents. Currently, no reports of molecules that have binding affinity to the C-terminal helical domain of survivin are available. As mentioned above, survivin forms a CPC with INCENP and Borealin; single anomalous dispersion (SAD) data have revealed that each specific amino acid sequence of INCENP and Borealin binds to the survivin protein (Figure 1). According to this report, we hypothesized that INCENP and Borealin protein segments can be scaffolds for targeting both monomer and dimer states of survivin. Here, we selected INCENP protein segments for a pilot study to develop survivin-targeting agents. Jeyaprakash et al have reported that the first 58 amino acids of INCENP can form a complex with survivin; several specific amino acid sequences directly interact electrostatically with survivin by the SAD method, as indicated in brown letters in Figure 1.26 We designed several INCENP-derived small peptides (INC peptides) that contain the amino acids directly interacting with survivin. In the present study, INC peptides were developed and evaluated as survivin-targeting agents for imaging and for cancer treatment.

2 MATERIALS AND METHODS

2.1 General information

All reagents were commercial products and were used without further purification, unless otherwise indicated. Fluorescein isothiocyanate isomer I was purchased from Sigma-Aldrich. N-9-fluorenylmethoxycarbonyl (Fmoc)-ε-Ahx-OH was obtained from Merck Millipore. Other Fmoc amino acids, HBTU, HOBt, and Fmoc-NH SAL resin were purchased from Watanabe Chemical Industries. Mass spectra were obtained with MALDI-time-of-flight mass spectrometry (TOF-MS) using Ultraflex MALDI-TOF/TOFMS (Bruker Daltonics). The HPLC analysis was undertaken using a Shimadzu HPLC system (LC-10AT pump with SPD-10A UV detector, λ = 254 nm). A Multi-mode Reader (Cytation3; Biotek) was used for measurement of absorbance and fluorescence.

2.2 Peptide synthesis

All peptides were synthesized by a stepwise solid-phase method using Fmoc chemistry with Fmoc-amino acid on 100150 mg Fmoc-NH-SAL resin (0.55 mmol amine/g resin). The resin was first soaked
for 18 hours in dimethylformamide (DMF). The Fmoc group was then deprotected with 20% (v/v) piperidine in DMF. After removing and washing out piperidine, Fmoc-amino acid (3 equiv), HBTU/HOBt (3 equiv), and N,N-diisopropylethylamine (DIPEA) (6 equiv) dissolved in DMF (1.2 mL) were added for the coupling reaction. For fluorescence labeling, FITC (3 equiv) and DIPEA (six equiv) were added to the protected resin-bound peptide in DMF and reacted for 18 hours. Following completion of labeling, the peptide was cleaved from the resin using TFA:H₂O:triisopropyl silane:1,2-ethanedithiol (94:2.5:1:2.5 v/v/v/v) for 90 minutes with shaking at room temperature. After separating the peptide from the resin, the filtrate was precipitated with chilled diethyl ether. The precipitate was centrifuged at 2500 g for 5 minutes, washed with diethyl ether 3 times, and centrifuged in between each washing step. The crude products were purified by HPLC on a Cosmosil C18 column (5C18-AR-II, 10 × 250 mm; Nacalai Tesque) using a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient at a flow rate of 2.0 mL/min. Each peptide was analyzed by MALDI-TOF-MS.

2.3 | Cell cultures

MDA-MB-231 cells (human breast cancer cells) and MCF-10A cells (human breast nontumorigenic epithelial cells) were purchased from ATCC. MIA PaCa-2 cells (human pancreatic cancer cells) were purchased from the Riken BioResource Research Center (BRC). MDA-MB-231 cells and MIA PaCa-2 cells were cultured in DMEM (low glucose) supplemented with 10% FBS. MCF-10A cells were grown in DMEM/F-12 supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 10 µg/mL insulin, and 0.5 µg/mL hydrocortisone. All media were supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in a humidified 5% CO₂ incubator at 37°C.

2.4 | Saturation binding assay of peptides to recombinant survivin protein

Recombinant human survivin was expressed and purified as previously described.27 Kinetic analyses were undertaken with the quartz crystal microbalance (QCM) system (Affinix Q; Initium). The survivin protein (40 µg/mL) was immobilized on the gold electrode of the sensor chip by amide bonds. The sensor chip was set on the QCM apparatus and soaked in buffer. An 8 µL aliquot of 10-256 µmol L⁻¹ INC peptide was sequentially injected into the cuvette. Frequency changes were monitored over time. The kinetic analysis was carried out by AQUA version 1.3 software (Initium).

2.5 | Confocal fluorescence imaging of cells with FITC-INC₁₁-₂₂

Cultured MDA-MB-231 and MCF-10A cells were fixed with formaldehyde and permeabilized with Triton X-100. The cells were then incubated with FITC-INC₁₁-₂₂ (10 µmol L⁻¹) for 2 hours and later washed with PBS. Immunofluorescence staining was carried out using mouse anti-survivin mAb (D8; Santa Cruz Biotechnology) as a primary Ab and Alexa Fluor 633 goat anti-mouse IgG(H + L) (Thermo Fisher Scientific Inc) as a secondary Ab. Fluorescence images were captured by a confocal laser scanning microscope (LSM710; Carl Zeiss) with excitation λ = 488 nm and emission λ = 494-601 nm for FITC, and excitation λ = 633 nm and emission λ = 639-758 nm for survivin.

2.6 | Western blot analysis

Cells were incubated with or without 1-50 µmol L⁻¹ of r9-INC₁₁-₂₂ for 48 hours, harvested, washed with PBS, and lysed for western blot analysis

FIGURE 1 Crystal structure of the survivin-Borealin-inner centromere protein (INCENP) core complex and the INCENP protein segment interacting with survivin. Brown characters indicate amino acids that directly bind to survivin through an electrostatic interaction. INCENP-derived small peptides (INC peptides) possessing sequences of INCENP protein segments (7-47) were designed as survivin-targeting agents. aCrystal structure referred from Protein Data Bank (PDB) (2QFA). bCrystal structure referred from PDB (1F3H).
to examine the expression levels of survivin and cleaved caspase-3. The equivalent protein was subjected to 15% SDS/PAGE, electrophoresed, and transferred on to a PVDF membrane. After blocking with 5% skim milk in TBS and washing, the membrane was incubated for 12 hours at 4°C with the following mAbs: mouse antisurvivin Ab (D8; Santa Cruz Biotechnology), mouse anticaspase-3 (3G2; Cell Signaling Technology), or mouse anti-β-actin Ab (2F1-1; BioLegend). After washing 3 times with PBS containing 0.1% Tween-20, the membrane was incubated with peroxidase-labeled secondary Ab (goat anti-mouse IgG-HRP; Santa Cruz Biotechnology). Chemiluminescent signals on the membranes were detected with the ChemiDoc Touch Imaging System (Bio-Rad).

2.7 | Cell proliferation assay

Cell proliferation was analyzed by MTT incorporation assay. Cells were plated on multiwell plates and treated with either the experimental compounds or the solvent 1% DMSO alone for 48 hours. MTT was added to the cells for 4 hours. Cells were then lysed in DMSO. Proliferation was assessed by measuring optical density readings at 570 nm (correction length, 650 nm) using Cytation3. Percentage viability was calculated by using 1% DMSO-treated cells as 100%.

2.8 | Flow cytometry

MIA PaCa-2 cells were plated into 6-well plates at 2 × 10^5 cells per well and cultured for 24 hours. Then the cells were treated with r9-INC16-22 (10 or 50 μmol L^{-1}) or the solvent 1% DMSO alone for 24 hours at 37°C. After trypsinization, cells were collected and centrifuged at 1000 g for 5 minutes. Following a PBS wash, cells were suspended in binding buffer. Cells were stained with annexin V-FITC/propidium iodide (PI) (MEBCYTO Apoptosis Kit; MBL Life Science) for 15 minutes in the dark. The cell pellets were analyzed by flow cytometry (BD Biosciences).

2.9 | Statistical analysis

The MTT assay of each cell line, western blot data of survivin protein in MIA PaCa-2 cells, and western blot data of cleaved caspase-3 were statistically analyzed using the Kruskal-Wallis test with Dunn’s multiple comparison post hoc test. Western blot data of survivin expression in MIA PaCa-2 cells were statistically analyzed using Student’s t test as shown. A P value less than .05 was considered statistically significant.

3 | RESULTS

3.1 | Synthesis and binding affinity assessment of INC peptides

Residues 7-47 of INCENP bind to survivin through an electrostatic interaction. Therefore, we designed 7- or 8-residue small peptides derived from INCENP protein segments (7-47) to discover scaffolds for new survivin-targeting agents. All peptides were synthesized by Fmoc solid-phase methods using HBTU/HOBt as coupling reagents. The crude peptides were purified by reversed-phase HPLC. The purified peptides were identified as target peptides by MALDI-TOF-MS (Table S1).

The binding affinities of the INC peptides to recombinant human survivin were evaluated by QCM; QCM is a highly sensitive and accurate mass measuring technique based on the linear decrease in resonance frequency in response to mass increase on a QCM plate at the nanogram level. This method has been used for the assessment of small molecule binding affinity to biomolecules. All INC peptides bound to survivin, indicating saturation and fitting well with the one binding site model (Figure S1A-F). The K_d values for each compound were calculated from the frequency changes of the QCM plate, which ranged from 91.4 to 255 nmol L^{-1}, and INC16-22 (DQKLMEF-NH_2) showed the highest binding affinity (K_d = 91.4 nmol L^{-1}) among the INC peptides, as illustrated in Table 1.

### TABLE 1

| INC peptides  | Sequence            | K_d (nmol L^{-1}) |
|---------------|---------------------|-------------------|
| INC15-12      | GPIHllel-NH_2       | 214 ± 46.3        |
| INC16-22      | DQKLMEF-NH_2       | 91.4 ± 4.86       |
| INC23-30      | LCNMDNKD-NH_2      | 117 ± 19.4        |
| INC27-34      | DNKDLVVL-NH_2      | 255 ± 23.0        |
| INC31-38      | LVWLEELIQ-NH_2     | 108 ± 14.7        |
| INC39-47      | EEAERMFTR-NH_2     | 135 ± 31.1        |

Note: Values are mean ± SEM (n = 3-5).
or therapy. First, we prepared a scrambled peptide, scINC16-22, containing the same amino acids as INC16-22 but in random order, to assess the sequence selectivity of INC16-22 to survivin (Table S2). Figure 2 shows the representative QCM sensorgram of the binding interactions between INC peptides and QCM electrodes coated with survivin. INC16-22 strongly responded to survivin in a dose-dependent manner, whereas scINC16-22 showed negligible response to survivin (Figure 2A). The binding affinity of scINC16-22 to survivin (K_d > 300 nmol L^-1) was much lower than that of INC16-22 (Table 2), indicating sequence selectivity of INC16-22 for survivin recognition. We then evaluated the response of INC16-22 to human serum albumin (HSA) to assess the binding interaction of INC16-22 with other proteins; no significant response between INC16-22 and HSA was observed (Figure 2B). These results indicate that INC16-22 selectively recognizes the survivin protein.

3.4 Fluorescence microscope imaging of FITC-INCl6-22

To assess whether INC16-22 derived peptides can be used as cancer diagnostic agents, we synthesized and identified a FITC-labeled INC16-22 derivative (FITC-INCl6-22), as shown in Table S1. The FITC-INCl6-22 showed comparable binding affinity for survivin (K_d = 66.0 nmol L^-1) as INC16-22 (Table 2). The cellular localization of FITC-INCl6-22 was examined using 3 cell lines (MDA-MB-231, MIA PaCa-2, and MCF-10A) showing varied protein expression levels of survivin. The MDA-MB-231 (human breast carcinoma cell line) and MIA PaCa-2 cells (human pancreatic carcinoma) are known to have high expression levels of survivin, whereas MCF-10A (human breast nontumorigenic epithelial cell line) has shown low expression levels of survivin. First, we confirmed the high expression of survivin in MDA-MB-231 and MIA PaCa-2 cells, whereas poor expression of survivin was detected in MCF-10A cells (Figure S2). The confocal microscopy images displayed significant FITC-INCl6-22 signal in the survivin-positive regions of MDA-MB-231 (Figure 3A) and MIA PaCa-2 cells (Figure S3). Nonetheless, negligible FITC-INCl6-22 fluorescent signals were observed in the MCF-10A cells (Figure 3B). Furthermore, quantitative line analysis indicated that FITC-INCl6-22 accumulation in cells correlated well with survivin expression levels (Figure 3C). However, the accumulation of FITC-INCl6-22 in MCF-10A cells was significantly lower compared with that in MDA-MB-231 cells (Figure 3D). These results indicate that FITC-INCl6-22 can recognize the cellular survivin protein.

3.5 Evaluation of r9-INCl6-22 as anticancer agents

Even small peptides may not readily penetrate cell membranes; therefore, we designed and synthesized cell-permeable o-Arg nonamer-fused INC16-22 (r9-INCl6-22), aiming to apply an intracellular

---

**TABLE 2** Dissociation constant (K_d) values of inner centromere protein-derived small peptide (INC)16-22 derivatives for survivin determined by quartz crystal microbalance assay

| INC peptides | Sequence | K_d (nmol L^-1) |
|--------------|----------|----------------|
| INC15-22     | CDQKLMEF-NH2 | 248 ± 61.0     |
| INC16-34     | DQKLMEFLCNMDKLVLNW-NH2 | 160 ± 40.3     |
| nnINC16-22   | DQKeLMEF-NH2   | 97.1 ± 24.3    |
| scINC16-22   | QLMDFKE-NH2   | >300           |
| FITC-INCl6-22| FITC-X-DQKLMEF-NH2 | 66.0 ± 11.9 |
| r9-INCl6-22  | Ac-r6-r5-r4-r3-X-DQKLMEF-NH2 | 68.2 ± 4.4 |
| r9-scINcl6-22 | Ac-r6-r5-r4-r3-X-QLDMFKENH2 | >1000 |

Note: Values are mean ± SEM (n = 3-5).

Abbreviations: cL, cycloleucine; X, 6-aminohexanoic acid.

-o-amino acids are denoted in lowercase.

---

**FIGURE 2** Kinetic analyses of the binding between survivin or human serum albumin (HSA) and inner centromere protein-derived small peptides (INC peptides) using the quartz crystal microbalance apparatus. Time course of the frequency change of the survivin-immobilized (A) or HSA-immobilized (B) sensor chip, responding to the addition of INC16-22 (red line) or scINC16-22 (black line). Various concentrations of INC16-22 were added to the cuvette. The resulting overall concentrations of INC16-22 in the cuvette were as follows: 1, 10 nmol L^-1; 2, 25 nmol L^-1; 3, 47.5 nmol L^-1; 4, 81.3 nmol L^-1; 5, 131.9 nmol L^-1; 6, 207.8 nmol L^-1; 7, 321.7 nmol L^-1; 8, 492.6 nmol L^-1; 9, 748.9 nmol L^-1
survivin-targeting agent. The synthesized r9-INCl6-22 was purified and identified as shown in Table S1. The QCM assay indicated that r9-INCl6-22 well-fitted the one binding site model (Figure S1J) and showed higher binding affinity to survivin ($K_d = 68.2$ nmol L$^{-1}$) compared with that of INC16-22. The r9-INCl6-22 strongly responded to survivin in a dose-dependent manner, whereas a scrambled peptide, r9-scINC16-22, showed negligible response to survivin (Table S1 and Figure S4A). There was no significant response between r9-INCl6-22 and HSA (Figure S4B). These results indicate that r9-INCl6-22 selectively recognizes the survivin protein, as in the case of INC16-22.

Next, we tested the ability of r9-INCl6-22 to inhibit cell proliferation by MTT assay. A small molecule survivin inhibitor, S12, was also used to compare the anticancer effects with r9-INCl6-22. Three cell lines (MDA-MB-231, MIA PaCa-2, and MCF-10A cells) were treated with 1, 10, or 100 µmol L$^{-1}$ r9-INCl6-22 for 48 hours. Interestingly, the inhibitory effect on cell proliferation by r9-INCl6-22 was quite different between the cancer cell lines. The proliferation of MDA-MB-231 cells was significantly inhibited (40% and 61% inhibition, respectively) at only high concentrations (10 and 100 µmol L$^{-1}$) of r9-INCl6-22 (Figure 4A). However, the proliferation of MIA PaCa-2 cells was significantly inhibited, even at 1 µmol L$^{-1}$ of r9-INCl6-22 (52% inhibition), as shown in Figure 4B. Similarly, S12 showed no proliferation inhibitory effect to MDA-MB-231 cells, whereas its inhibition was significantly decreased in the cells treated with r9-INCl6-22 at 10 µmol L$^{-1}$, compared to the untreated cells (Figure 6). Next, we evaluated whether r9-INCl6-22 showed apoptosis and necrosis effects on cancer cells. MIA PaCa-2 cells were treated with r9-INCl6-22 for 24 hours, stained with annexin V-FITC/PI, and examined by flow cytometry. As shown in Figure 7, the percentage of early apoptosis rates were 52.4% and
49.6% when MIA PaCa-2 cells were treated with 10 and 50 μmol L⁻¹ r9-INCl₆-2₂, respectively, compared with the control groups. The late apoptosis and necrosis rates of the cells were 29.4% and 44.2% at 10 and 50 μmol L⁻¹ r9-INCl₆-2₂, respectively. These results suggest that r9-INCl₆-2₂ showed cytotoxicity to cancer cells by inducing apoptosis.

4 | DISCUSSION

Survivin is highly expressed in most cancer types but is undetectable in nondividing tissues.3,4,6 Accordingly, survivin has been considered an ideal target for the specific diagnosis and treatment of cancers. Various survivin-targeting agents have been developed, such as...
molecules and antisense oligonucleotides that suppress survivin expression, destabilize the survivin protein, or disrupt interactions with other oncoproteins. However, there are currently no available survivin-targeting agents for the diagnosis and treatment of cancer in clinical practice.

We discovered novel survivin-targeting peptides, derived from the INCENP protein sequence, that directly interact with the survivin protein. Our study provides a proof of concept that the C-terminal helical coiled-coil domain of survivin can be a prospective target site for drug development. In our study, short peptide INC_{16-22}, consisting of only 7 amino acids, showed the highest binding affinity to survivin. Although the reason is unclear, it is suggested that residues 16-22 of the INCENP protein are especially important for the binding to survivin. Our study provided valuable information for the further development of coiled-coil drug discovery targets for survivin. Confocal microscope images showed fluorescence signals of FITC-labeled INC_{16-22} corresponding to the expression of survivin protein in the cells (Figures 3 and S3), indicating that INC peptides can be applied to survivin-targeting cancer imaging agents. There was a partly inconsistent accumulation of FITC-INC_{16-22} in the survivin-expressing regions in MDA-MB-231 (Figure 3) and MIA PaCa-2 cells (Figure S3), which might be due to lower binding affinity of the peptide ($K_d = 66.0 \text{ nmol L}^{-1}$) for the survivin protein.

Both dimer and monomer survivin can inhibit caspase-dependent apoptosis. Small molecules that bind the survivin dimerization interface, such as S12 and LQZ-7F, can be prospective candidate anticancer agents; however, these ligands cannot intrinsically bind to the monomer survivin. As the putative binding site of INC peptides is located on the C terminus $\alpha$-helix region of survivin, these peptides could bind to both dimer and monomer survivin. Regarding this aspect, INC peptides are superior to binders to the dimerization interface of survivin. Indeed, r9-INC_{16-22} showed relatively higher antiproliferative effects in cancer cells than those of S12 (Figure 4). Consistent with previous reports, our study also indicated a difference in the antiproliferative activity of r9-INC_{16-22} between MDA-MB-231 and MIA PaCa-2 cells. The antiproliferative effect of LQZ-7F on cancer cells has been associated with survivin protein levels. In this study, r9-IN_{16-22} showed a stronger antiproliferative effect on MIA PaCa-2 cells than on MDA-MB-231 cells. Survivin protein levels were consistently reduced in MIA PaCa-2 cells, but not in MDA-MB-231 cells (Figure 5). It has been reported that survivin dimer site ligands cause degradation of survivin by the proteasome or by autophagy; it is unclear why r9-IN_{16-22}
induced stronger survivin degradation in MIA PaCa-2 cells than in MDA-MB-231 cells; however, the stability of survivin in regards to the proteasome could be cell-type specific. On the contrary, we observed significant apoptotic effects in response to treatment with r9-INC_{16-22} at 10 and 50 μmol L⁻¹ concentrations, with increased expression of cleaved caspase-3 (Figure 6) and increased number of annexin V-FITC/PI-positive cells in MIA PaCa-2 cells (Figure 7). However, treatment with 40 μmol L⁻¹ r9-INC_{16-22} could not induce significant survivin degradation in MIA PaCa-2 cells. Therefore, the cytotoxicity of r9-INC_{16-22} might be caused mainly by the disruption of the survivin-mediated antiapoptosis function and partly because of the direct degradation of the survivin protein. The antiproliferative effects of r9-INC_{16-22} could be responsible for inhibiting the stabilization of XIAP as well as the apoptotic effects of Smac/DIABLO that can induce the production of cleaved caspase-3.⁷⁻⁹ In addition, Smac/DIABLO. Clearly, further studies are needed to develop INC peptide derivatives with special amino acids, such as unusual and nonnatural amino acids or peptidomimetics, to overcome the metabolic stability. Nevertheless, we have discovered a novel scaffold for the development of peptide-based survivin-targeting molecules for clinically available agents for cancer diagnosis and treatment.

In conclusion, we designed and synthesized small 7-19 residue peptides as survivin-targeting agents for cancer imaging and therapy. Among them, INC_{16-22} showed the strongest binding affinity (91.4 nmol L⁻¹) to the survivin protein; FITC-INC_{16-22} showed consistent localization with survivin expression levels in cell lines. Exposure to r9-INC_{16-22} showed antiproliferative effects with pronounced apoptosis in MIA PaCa-2 cells. Our findings suggest that INC peptides can be useful scaffolds for novel cancer theranostic agents.

ACKNOWLEDGMENTS

Financial support was provided by a Grant-in-Aid for Young Scientists (B) (Grant No. 21791180), Grant-in-Aid for Scientific Research (C) (Grant No. 15K09895) from the Japan Society for the Promotion of Science (JSPS), and Grant-in-Aid for JSPS Research Fellow (Grant No. 17J11488). This work was supported in part by the Takeda Science Foundation, Konica Minolta Science and Technology Foundation, Mochida Memorial Foundation for Medical and Pharmaceutical Research, SGH Foundation, and Terumo Life Science Foundation.

DISCLOSURE

The authors declare that they have no competing interests.

ORCID

Takeshi Fuchigami https://orcid.org/0000-0001-8141-1212

REFERENCES

1. Altieri DC. Survivin and IAP proteins in cell-death mechanisms. Biochem J. 2010;430:199-205.
2. Li F, Ambrosini G, Chu EY, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature. 1998;396:580-584.
3. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med. 1997;3:917-921.
4. Fukuda S, Pelus LM. Survivin, a cancer target with an emerging role in normal adult tissues. Mol Cancer Ther. 2006;5:1087-1098.
5. Mitani AC, Mita MM, Nowrooki ST, Giles FJ. Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. Clin Cancer Res. 2008;14:5000-5005.
6. Velculescu VE, Madden SL, Zhang L, et al. Analysis of human transcriptomes. Nat Genet. 1999;23:387-388.
7. Dohi T, Okada K, Xia F, et al. An IAP-IAP complex inhibits apoptosis. J Biol Chem. 2004;279:34087-34090.
8. Ceballos-Cancino G, Espinosa M, Maldonado V, Melendez-Zajgla J. Regulation of mitochondrial Smad/DIABLO-selective release by survivin. Oncogene. 2007;26:7569-7575.
9. Song Z, Yao X, Wu M. Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. J Biol Chem. 2003;278:23130-23140.
10. Li D, Hu C, Li H. Survivin as a novel target protein for reducing the proliferation of cancer cells (review). Biomed Rep. 2016;8:399-406.
11. Carmena M, Wheelock M, Funabiki H, Earnshaw WC. The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. Nat Rev Mol Cell Biol. 2012;13:789-803.
12. Khan Z, Khan AA, Yadav H, Prasad G, Bisen PS. Survivin, a molecular target for therapeutic interventions in squamous cell carcinoma. Cell Mol Biol Lett. 2017;22:8.
13. Chen X, Duan N, Zhang C, Zhang W. Survivin and tumorigenesis: molecular mechanisms and therapeutic strategies. J Cancer. 2016;7:314-323.
14. Peery RC, Liu JY, Zhang JT. Targeting survivin for therapeutic discovery: past, present, and future promises. Drug Discov Today. 2017;22:1466-1477.
15. Garg H, Suri P, Gupta JC, Taiwar GP, Dubey S, Dubey S. Survivin: a unique target for tumor therapy. Cancer Cell Int. 2016;16:49.
16. Iwasa T, Okamoto I, Suzuki M, et al. Radiosensitizing effect of YM155, a novel small-molecule survivin suppressant, in a wide variety of human cancer cell lines and xenograft models. Cancer Sci. 2016;107:649-650.
17. Mehta A, Zhang L, Bourfoua M, et al. Inhibition of survivin with YM155 induces durable tumor response in anaplastic thyroid cancer. Clin Cancer Res. 2015;21:4123-4132.
18. Nakahara T, Kita A, Yamanaka K, et al. Broad spectrum and potent antitumor activities of YM155, a novel small-molecule survivin suppressant, in a wide variety of human cancer cell lines and xenograft models. Cancer Sci. 2011;102:614-621.
19. Kelly RJ, Thomas A, Rajan A, et al. A phase I/II study of sepantronium bromide (YM155, survivin suppressor) with paclitaxel and carboplatin in patients with advanced non-small-cell lung cancer. Ann Oncol. 2013;24:2601-2606.
20. Kudchadkar R, Ernst S, Chmieleowski B, et al. A phase 2, multicenter, open-label study of sepantronium bromide (YM155) plus docetaxel in patients with stage III (unresectable) or stage IV melanoma. Cancer Med. 2015;4:643-650.
21. Clemens MR, Gladkov OA, Gartner E, et al. Phase II, multicenter, open-label, randomized study of YM155 plus docetaxel as first-line treatment in patients with HER2-negative metastatic breast cancer. Breast Cancer Res Treat. 2015;149:171-179.
22. Glaros TG, Stockwin LH, Mullendore ME, Smith B, Morrison BL, Newton DL. The “survivin suppressors” NSC 80467 and YM155 induce a DNA damage response. Cancer Chemother Pharmacol. 2012;70:207-212.
23. Feng W, Yoshida A, Ueda T. YM155 induces caspase-8 dependent apoptosis through downregulation of survivin and Mcl-1 in human leukemia cells. Biochem Biophys Res Commun. 2013;435:52-57.

24. Ansell SM, Arendt BK, Grote DM, et al. Inhibition of survivin expression suppresses the growth of aggressive non-Hodgkin’s lymphoma. Leukemia. 2004;18:616-623.

25. Rodel F, Frey B, Leitmann W, Capalbo G, Weiss C, Rodel C. Survivin antisense oligonucleotides effectively radiosensitize colorectal cancer cells in both tissue culture and murine xenograft models. Int J Radiat Oncol Biol Phys. 2008;71:247-255.

26. Wiechno P, Somer BG, Mellado B, et al. A randomised phase 2 study combining LY2181308 sodium (survivin antisense oligonucleotide) with first-line docetaxel/prednisone in patients with castration-resistant prostate cancer. Eur Urol. 2014;65:516-520.

27. Berezov A, Cai Z, Freudenberg JA, et al. Disabling the mitotic spindle and tumor growth by targeting a cavity-induced allosteric site of survivin. Oncogene. 2012;31:1938-1948.

28. Chettiar SN, Cooley JV, Park I-H, et al. Design, synthesis and biological studies of survivin dimerization modulators that prolong mitotic cycle. Bioorg Med Chem Lett. 2013;23:5429-5433.

29. Guvenc H, Pavlyukov MS, Joshi K, et al. Impairment of glioma stem cell survival and growth by a novel inhibitor for Survivin-Ran protein complex. Clin Cancer Res. 2013;19:631-642.

30. Qi J, Dong Z, Liu J, et al. Effective targeting of the survivin dimerization interface with small-molecule inhibitors. Cancer Res. 2016;76:453-462.

31. Fuchigami T, Mizoguchi T, Ishikawa N, et al. Synthesis and evaluation of a radioiodinated 4,6-diaryl-3-cyano-2-pyridinone derivative as a survivin targeting SPECT probe for tumor imaging. Bioorg Med Chem Lett. 2016;26:999-1004.

32. Ishikawa N, Fuchigami T, Mizoguchi T, Yoshida S, Haratake M, Nakayama M. Synthesis and characterization of radioiodinated 3-phenethyl-2-indolinone derivatives for SPECT imaging of survivin in tumors. Bioorg Med Chem. 2018;26:3111-3116.

33. Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, Noel JP. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. Nat Struct Biol. 2000;7:602-608.

34. Chantalat L, Skoufias DA, Kleman JP, Jung B, Dideberg O, Margolis RL. Crystal structure of human survivin reveals a bow tie-shaped dimer with two unusual alpha-helical extensions. Mol cell. 2000;6:183-189.

35. Pavlyukov MS, Antipova NV, Balashova MV, Vinogradova TV, Kopantzev EP, Shakhparonov MI. Survivin monomer plays an essential role in apoptosis regulation. J Biol Chem. 2011;286:23296-23307.

36. Jeyaprakash AA, Klein UR, Lindner D, Ebert J, Nigg EA, Conti E. Structure of a Survivin-Borealin-INCENP core complex reveals how chromosomal passengers travel together. Cell. 2007;131:271-285.

37. Kuramochi K, Miyano Y, Enomoto Y, et al. Identification of small molecule binding molecules by affinity purification using a specific ligand immobilized on PEGA resin. Bioconjug Chem. 2008;19:2417-2426.

38. Yang L, Cao Z, Yan H, Wood WC. Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. Cancer Res. 2003;63:6815-6824.

39. Kubota H. Quality control against misfolded proteins in the cytosol: a network for cell survival. J Biochem. 2009;146:609-616.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Fuchigami T, Ishikawa N, Nozaki I, et al. Discovery of inner centromere protein-derived small peptides for cancer imaging and treatment targeting survivin. Cancer Sci. 2020;111:1357–1366. https://doi.org/10.1111/cas.14330