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Identification of small molecule inhibitors of p27^{Kip1} ubiquitination by high-throughput screening

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Dysregulation of p27^{Kip1} due to proteolysis that involves the ubiquitin ligase (SCF) complex with S-phase kinase-associated protein 2 (Skp2) as the substrate-recognition component (SCF^{Skp2}) frequently results in tumorigenesis. In this report, we developed a high-throughput screening system to identify small-molecule inhibitors of p27^{Kip1} degradation. This system was established by tagging Skp2 with fluorescent monomeric Azami Green (mAG) and CDK subunit 1 (Cks1) (mAGSkp2–Cks1) to bind to p27^{Kip1} phosphopeptides. We identified two compounds that inhibited the interaction between mAGSkp2–Cks1 and p27^{Kip1}: linichlorin A and gentian violet. Further studies have shown that the compounds inhibit the ubiquitination of p27^{Kip1} *in vitro* as well as p27^{Kip1} degradation in HeLa cells. Notably, both compounds exhibited preferential antiproliferative activity against HeLa and tsFT210 cells compared with NIH3T3 cells and delayed the G_1 phase progression in tsFT210 cells. Our approach indicates a potential strategy for restoring p27^{Kip1} levels in human cancers. (*Cancer Sci* 2013; 104: 1461–1467)

Cyclin-dependent kinases (CDK), which function upon activation by cyclin binding, are known to be an active key molecule complexes that regulate the progression of cell cycles. The activities of CDK are constrained by CDK inhibitors (CKI).[1–3] One CKI, p27^{Kip1}, a 198-amino-acid protein that was discovered in cells arrested by TGF-β,[4,5] has significant functions in governing cell proliferation, cell motility, senescence and apoptosis.[1–3] Unlike other tumor suppressors, for instance p53, p27^{Kip1} is rarely mutated in human cancers. It is reported that p27^{Kip1} is usually dysregulated in cancers, despite high or constant p27^{Kip1} mRNA levels.[3,6,7] During the cell cycle, p27^{Kip1} negatively regulates the G_1-S transition, and its levels peak during G_1 phase, causing arrest in G_1.[1,3,8,9] These fluctuations are not mirrored in its mRNA levels,[9,10] suggesting that downregulation of p27^{Kip1} in human cancers, which is associated with many aggressive phenotypes and a poor prognosis in various cancers (e.g. breast, colon, prostate, lung and gastric cancers), is caused primarily by post-transcriptional events.[3,9]

It is well known that p27^{Kip1} is degraded through a sequential degradation system, called the ubiquitin-proteasome system (UPS).[11] This process begins with the transfer and covalent attachment of ubiquitin to target proteins through a cascade of enzymatic reactions, followed by degradation of the marked target proteins by the proteasome.[12] Biochemical studies have shown that p27^{Kip1} is ubiquitinated *in vitro* and *in vivo* primarily by SCF^{Skp2}, a ubiquitin ligase complex that contains S-phase kinase-associated protein 2 (Skp2).[13–16] p27^{Kip1} is recognized by Skp2 only when it is phosphorylated by CDK2/cyclin E on Thr-187.[17–19] Moreover, recognition of p27^{Kip1} by SCF^{Skp2} requires an accessory protein, CDK subunit 1 (Cks1), which binds to phosphorylated p27^{Kip1} and Skp2.[20,21] Thus, Skp2 and Cks1 are equally important for the recognition of and binding to p27^{Kip1}.

Several published studies suggest that Skp2 has oncogenic activity.[22,23] Notably, transformed cell lines and human cancers are frequently associated with overexpression of Skp2 and p27^{Kip1} downregulation.[6,24,25] With activated Ras, Skp2 transforms cells[26] and induces lymphomas in transgenic mice,[27] and transgenic expression of Skp2 in mouse prostate causes hyperplasia, dysplasia and low-grade prostate carcinoma with significant p27^{Kip1} downregulation.[28] In contrast, Skp2 knockout mice are fertile and viable and have elevated p27^{Kip1} levels.[16] Moreover, siRNA-mediated silencing of Skp2 in oral and lung carcinomas inhibits and suppresses tumor proliferation.[29,30] Thus, the involvement of Skp2 in many aggressive cancers suggests that targeting it using small-molecule inhibitors is a promising cancer therapy.

Recent advances in chemical biology studies have allowed us to identify such biologically active small molecule inhibitors of various targets by high-throughput compound screening. Several compounds able to prevent p27^{Kip1} degradation are reported.[31–33] In Rico-Bautista *et al.*,[31] cell-based high-throughput screening using Skp2 overexpressing cells identified two compounds that restored the levels of nuclear p27^{Kip1} efficiently. While the direct molecular targets of these compounds were yet to be unveiled, one of the compounds turned out to induce the downregulation of Skp2 in cells. The other compounds of this study[31] and a compound found in another study[32] have an activity to increase the mRNA level of p27^{Kip1}. Using an *in vitro*-reconstituted electrophoresis-based ubiquitination assay as a screening system, a compound that inhibits ubiquitination dependent proteolysis of p27^{Kip1} is isolated.[33]

In this report, we developed a high-throughput screening (HTS) system to identify small molecule inhibitors of protein–protein interaction between Skp2–Cks1 and p27^{Kip1}. We identified two small molecule inhibitors of p27^{Kip1} degradation. These small molecule inhibitors inhibited the *in vitro* ubiquitination of p27^{Kip1} by SCF^{Skp2}, stabilized p27^{Kip1} levels in HeLa cells and inhibited the growth of human and mouse cancer cells.

Materials and Methods

Recombinant baculoviruses. To construct recombinant baculoviruses that express mAGSkp2 (mAG, N-terminally fused fluorescent monomeric Azami Green)[34] and Cks1, hemagglutinin (HA)-tagged mAGSkp2 and Cks1 DNA fragments were inserted into the *Eco*RI-*Not*I and *Sal*I-*Not*I sites of pFastBac. The recombinant plasmids were then transformed into

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DH10Bac cells followed by transfection of the obtained recombinant bacmids into ExpressSF+ Serum-Free Insect Cells (Protein Sciences) using Cellfectin II (Invitrogen, Carlsbad, CA, USA). Recombinant baculovirus that expressed Skp1 was obtained from Dr Keiichi Nakayama (Kyushu University, Fukuoka-shi, Japan).

Antibodies. For western blot analysis to detect endogenous protein levels, commercial antibodies against Skp2 (Santa Cruz), p130 (Santa Cruz) and p21 (Abcam, Cambridge, UK) were used at concentrations recommended by these manufacturers.

Cell culture conditions. Insect cells were grown in Sf-900 II SFM (serum-free medium complete 1×, GIBCO) and cultured at 27°C with constant stirring at 130 rpm. A total of 150 mL of insect cell culture was coinfected with 3 mL of each recombinant baculovirus (mAGSkp2, Cks1 and Skp1) for 72 h before the cells were harvested. HeLa cells were maintained in DMEM (Invitrogen) that contained 10% fetal calf serum, and NIH3T3 cells were maintained in MEM (SIGMA, St. Louis, MO, USA) that contained 10% calf serum; both media were supplemented with 0.5% penicillin/streptomycin (Invitrogen). Cells were maintained in a humidified incubator at 37°C and 5% CO_2. The tsFT210 cell line, which is a temperature-sensitive mutant that has been isolated from the mouse mammary carcinoma cell line FM3A, was maintained in RPMI-1640 medium (Invitrogen) that was supplemented with 5% calf serum in a suspension culture at 32°C and 5% CO_2. tsFT210 cells can be arrested at G2 phase and are large in size when cultured at 39°C at 5% CO_2.[35–37]

Development of binding assay and screening for inhibitors of Skp2–Cks1 and p27^{Kip1} interaction. A 24-residue (residue 175–197) p27^{Kip1} phosphopeptide that harbored the target sequence (C-SDGSPNAGSVEQpTPKKPGLRRRQT), which binds Skp1–Skp2–Cks1 with equal affinity compared with the full-length phosphorylated p27^{Kip1} peptide[38] and its derivatives without phosphorylation, was chemically synthesized, purified and bound covalently to maleimide-activated 96-well plates (Pierce) at 100 μL/well of a 10 μM solution according to the manufacturer's protocol.

Insect cells expressing mAGSkp2, Cks1 and Skp1 were lysed by sonication in lysis buffer (20 mM Tris, pH 7.5, 125 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM EDTA, 200 μM Na_3VO_4 and 1 mM dithiothreitol), containing Complete Protease Inhibitors (Roche Applied Science, Penzberg, Germany). Screening for inhibitors of the Skp2–Cks1 and p27^{Kip1} interaction from RIKEN NPDepo (Natural Product Depository) chemical library[39] was performed at high-throughput manner on a Biomek 2000 liquid handling system (Beckman-Coulter, Brea, CA, USA), essentially as described previously.[40] Briefly, 50 μL of insect cell lysate was mixed with chemical compounds at a final concentration of 60 μg/mL. The mixtures were added to 96-well phosphopeptide-bound plates and incubated overnight at 4°C on a shaker. After five rinses with 0.05% Nonidet P-40 in PBS and two rinses with PBS, the reaction was quantified spectrophotometrically at excitation 488 nm and emission 546 nm.

Cell proliferation assay. A total of 4×10^3 HeLa cells/200 μL/well, 1×10^4 NIH3T3 cells/200 μL/well or 1.6×10^4 tsFT210 cells/200 μL/well were seeded into the

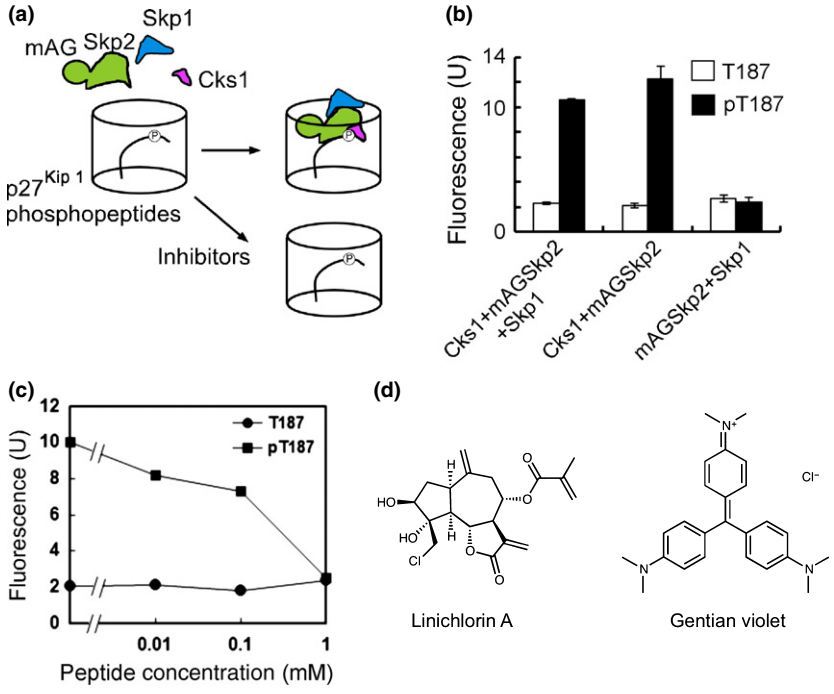

Fig. 1. Screening system and high-throughput screening for inhibitors of Skp2–Cks1 and p27^{Kip1} interaction. (a) Insect cell lysates expressing Skp1, mAGSkp2 and Cks1 were added to the wells of 96-well plates that were covalently attached with p27^{Kip1} phosphopeptides. Bound Skp1–mAGSkp2–Cks1 to p27^{Kip1} phosphopeptides in a phosphorylation-dependent manner was confirmed by spectrofluorometry. When inhibitors are present, the fluorescent signal by mAGSkp2 decreases. (b) p27^{Kip1} phosphopeptide binding sequence to Skp1–Skp2–Cks1 (pT187; C-SDGSPNAGS-VEQpTPKKPGLRRRQT) or its nonphosphorylated version (T187) was bound to 96-well plates at 100 μL/well of 10 μM peptide solution. Binding of Skp1–mAGSkp2–Cks1 to p27^{Kip1} peptides was detected spectrofluorometrically and was only observed with pT187, indicated by the black bars. No binding was detected with T187 (white bars). Binding was observed when Cks1 was present, whereas no effect was seen with Skp1 in the binding assay. Data are representative of four replicates. (c) Peptide competitive binding assay. Insect cell lysates expressing mAGSkp2 and Cks1 mixed with p27^{Kip1} phosphopeptide solution at the indicated concentrations were added to 96-well plates covalently bound with p27^{Kip1} phosphopeptides. Using a spectrophotometer, a concentration-dependent decrease in binding was observed with the p27^{Kip1} phosphopeptide solution. At 1 mM phosphopeptide solution, binding was abolished. (d) Structures of linichorin A and gentian violet. These compounds were identified as small molecule inhibitors of Skp2–Cks1 and p27^{Kip1} interaction.
[Correction added on 23 October 2013, after first online publication: In Figure 1d, the structure of linichlorin A is not correct in the first online publication. In this version of the article, it has been replaced with the correct structure.]

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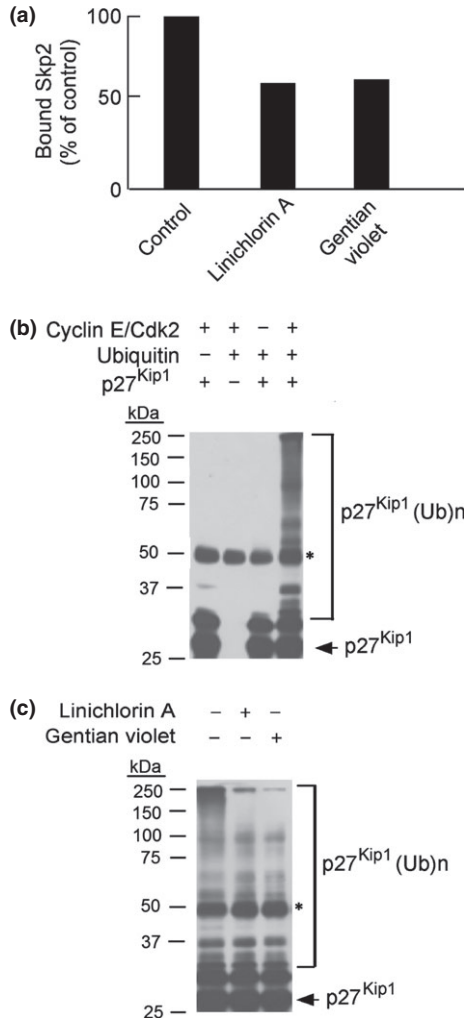

Fig. 2. Compounds inhibit *in vitro* ubiquitination of $p27^{Kip1}$. (a) Effect of compounds on the interaction between Skp2–Cks1 complex and phosphorylated $p27^{Kip1}$. Skp2–Cks1 complex was pulled down by sepharose beads conjugated with p27-derived phosphopeptides or non-phophopeptides. Bound complex was examined by western blot with anti-HA. Binding was reduced in the presence of compounds. (b) Ubiquitination reactions were subjected to SDS-PAGE, followed by immunoblot analysis with anti-$p27^{Kip1}$, to examine the effect of the indicated reagents and enzymes on *in vitro* ubiquitination of $p27^{Kip1}$. *Signal derived from IgG heavy chain. (c) Effect of compounds on *in vitro* ubiquitination of $p27^{Kip1}$. In the presence of linichlorin A at 3.2 μM and gentian violet at 0.4 μM, *in vitro* ubiquitination of $p27^{Kip1}$ was inhibited (concentrations represent the IC_{50} of each compound in HeLa cells; refer to Fig. 4a for details). Quantification by ImageQuant TL showed a significant reduction of 70–80% in the amount of *in vitro* ubiquitinated $p27^{Kip1}$ by both compounds. *Signal derived from IgG heavy chain.

wells of 96-well plates and were incubated for 24 h in a humidified 37°C incubator with 5% CO_2 for HeLa and NIH3T3 cells and in a 32°C incubator for tsFT210 cells. Then, the cells were treated with increasing concentrations of compounds for 48 h, cell growth was measured using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan), and IC_{50} values were calculated, based on the absorbance at 450 nm (Wallac 1420 ARVO; PerkinElmer, Waltham, MA, USA).

Protein stability assay. HeLa cells were treated with compounds at their IC_{50} concentrations for 18 h, and 20 μg/mL cycloheximide (CHX) was added for the indicated times prior to the preparation of cell lysates. Total protein amounts were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), with BSA as standard. Approximately 40 μg of cell lysate was subjected to SDS-PAGE, followed by immunoblot analysis with anti-$p27^{Kip1}$ (BD Transduction Laboratory, Franklin Lakes, NJ, USA).

RNA extraction, cDNA synthesis and semi-quantitative RT-PCR. Total RNA of compound-treated HeLa cells was extracted using TRizol (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega, Fitchburg, WI, USA) and was used for semi-quantitative PCR employing intron-spanning primers.[41] Primer sequences for $p27^{Kip1}$ are FW: 5′-ACCTGCAACCGACGATTCTT-3′ and RV: 5′-CCC TTCCCCAAAATTGCTTC-3′. Primer sequences for β-actin are FW: 5′-CTGGACTTCGAGCAAGA-3′ and RV: 5′-TCC TGCTTGCTGATCCA-3′.

Purification of $p27^{Kip1}$. The coding region of $p27^{Kip1}$ was cloned into the pET-28a(+) vector with 8 His-Tag at the N-terminal, using the *Bam*HI and *Nco*I sites to produce recombinant $p27^{Kip1}$. Recombinant $p27^{Kip1}$ was transformed and expressed in BL21 (DE3) cells and purified with HisTrap affinity columns (GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocols.

***In vitro* ubiquitination assay.** The $p27^{Kip1}$ *in vitro* ubiquitination assay was performed as previously described[21,42] using SCF complex expressed and immunoprecipitated from HEK293T cells.

Flow cytometric analysis. To obtain G_2-synchronized cells, tsFT210 cells were seeded into the wells of 24-well plates at 10×10^4 cells/500 μL/well and preincubated at 39°C for 18 h. Then, the cells were treated with compounds at the indicated concentrations prior to incubation at 32°C for the indicated times. Cell suspensions were harvested, washed with PBS, and resuspended in 500 μL propidium iodide buffer, containing 50 μg/mL propidium iodide (Sigma-Aldrich, St. Louis, MO, USA), 0.1% sodium citrate, 0.2% Nonidet P-40 and 2 μg/mL RNase A (Nacalai Tesque), for at least 30 min in the dark. The DNA content of the cells was analyzed on a Cytomics FC500 (Beckman Coulter).

Results

Development of screening system to identify inhibitors of Skp2–Cks1 and $p27^{Kip1}$ interaction. We developed a high-throughput screening system using Skp1, Skp2 and Cks1 expressed in the baculovirus protein expression system and phosphopeptides of $p27^{Kip1}$-derived sequences. In this system, we fused mAG, a fluorescent protein from the stony coral *Galaxeidae*[34] to the N-terminus of Skp2, allowing us to quantify the binding of mAGSkp2–Cks1 to $p27^{Kip1}$ by spectrofluorometry (Fig. 1a).

When insect cell lysates expressing Skp1, mAG-Skp2 and Cks1 were incorporated into the 96-well plates in which $p27^{Kip1}$-derived phosphopeptides were covalently bound, the binding of mAG-Skp2 to $p27^{Kip1}$ peptides was detected by spectrofluorometry. The binding was observed only to $p27^{Kip1}$ phosphopeptides (Fig. 1b). The importance of Skp1 and Cks1 in $p27^{Kip1}$ phosphorylation-dependent binding was also evaluated and we noted that Skp1 is not necessary for $p27^{Kip1}$ phosphorylation-dependent binding. In contrast, the fluorescent signal was abolished in cell lysates that did not express Cks1. Thus, Cks1 but not Skp1 is essential for $p27^{Kip1}$ phosphopeptide recognition (Fig. 1b).

To determine the reliability of our screening system, we performed a peptide competitive binding assay. Insect cell lysates expressing mAG-Skp2 and Cks1, mixed with $p27^{Kip1}$ phosphopeptide solution at the indicated concentrations, were added to 96-well plates to which $p27^{Kip1}$ phosphopeptides were bound

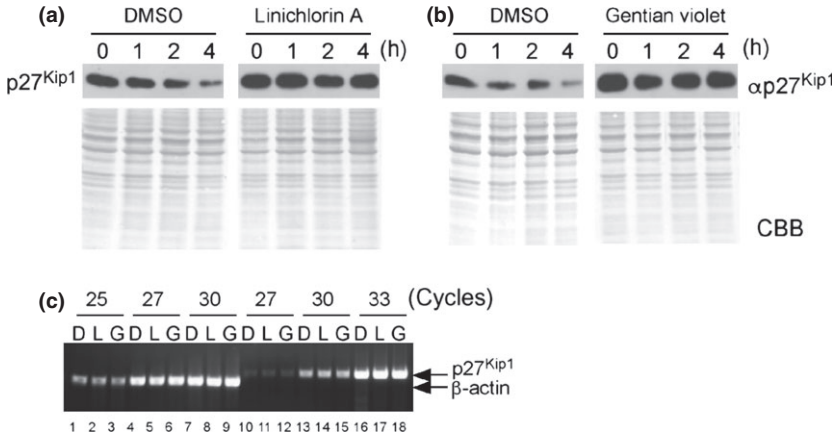

Fig. 3. Stabilization of $p27^{Kip1}$ in HeLa cells by the compounds. Exponentially growing HeLa cells were treated with linichlorin A at 3.2 μM (a) or gentian violet at 0.4 μM (b) for 18 h (concentrations represent the IC_{50} of each compound in HeLa cells; refer to Fig. 4a for details); 20 μg/mL CHX was then added, and $p27^{Kip1}$ levels were measured at the indicated times by western blot with anti-$p27^{Kip1}$. Coomassie Brilliant Blue (CBB) stain is also shown in the lower panel of each immunoblot. (c) Expression levels of mRNA after compound treatment were examined by RT-PCR (25, 27 and 30 cycles for mRNA detection of β-actin [lanes 1–9] and 27, 30 and 33 cycles for that of $p27^{Kip1}$ [lanes 10–18], respectively). HeLa cells were treated with DMSO (D), linichlorin A (L) (3.2 μM) and gentian violet (G) (0.4 μM) for 18 h, and RNA was extracted and processed for semi-quantitative RT-PCR.

(Fig. 1c). In the presence of 10 μM phosphopeptides, the interaction between mAG-Skp2–Cks1 and well-bound $p27^{Kip1}$ phosphopeptides was inhibited by approximately 20%. This binding decreased further with higher concentrations of the $p27^{Kip1}$ phosphopeptide solution, indicating the reliability and efficacy of our screening system. Thus, we used this system for small-molecule inhibitor screening.

Identification of linichlorin A and gentian violet as specific inhibitors of Skp2–Cks1 and $p27^{Kip1}$ interaction. We screened approximately 20 000 compounds in the RIKEN NPDepo chemical library at 60 μg/mL.[39] We identified 258 compounds that reduced the fluorescence of mAGSkp2 to <80% of control levels as primary hits. In a secondary screening, all 258 hit compounds were re-examined at the same concentration to confirm reproducibility of their inhibitory activities and, at the same time, compounds that inhibit other phosphorylation dependent protein interactions, such as polo box domain dependent interaction,[40] were excluded. After these selections, 30 compounds were considered positive in our assay. Next, we measured the effects of these compounds on cell growth. Among the 30 compounds, 15 compounds showed strong growth inhibition on HeLa cells while the other 15 compounds exhibited weaker growth inhibition. Among the 15 stronger compounds, linichlorin A and gentian violet were found to show the strongest effect on cell growth (Fig. 1d). We also observed that the inhibitory activity of linichlorin A and gentian violet obtained from the pulled down assay (Fig. 2a) corresponds to the inhibitory activity obtained in HTS. Thus, these two compounds were selected for further study.

Linichlorin A and gentian violet inhibit *in vitro* ubiquitination of $p27^{Kip1}$. As shown in Figure 2(b), we have constructed the *in vitro* ubiquitination reaction of $p27^{Kip1}$. The ubiquitination was observed only when the reagents and enzymes (ubiquitin, $p27^{Kip1}$ and CDK2/Cyclin E) were present in the reaction. After adding linichlorin A or gentian violet to the ubiquitination reaction, *in vitro* $p27^{Kip1}$ ubiquitination declined by 70–80% (Fig. 2c), as quantified on an ImageQuant TL (GE Healthcare). Thus, linichlorin A and gentian violet inhibited $p27^{Kip1}$ ubiquitination *in vitro*.

$p27^{Kip1}$ stabilization by linichlorin A and gentian violet. To examine the effect of these compounds on the stability of $p27^{Kip1}$, exponentially growing HeLa cells were treated with the compounds for 18 h. Then, 20 μg/mL CHX was administered to the cells to block the protein translation, and $p27^{Kip1}$ levels were measured by immunoblotting. As shown in Figure 3(a) and (b), $p27^{Kip1}$ was degraded rapidly after the addition of CHX in control cells (DMSO). In contrast, the degradation of $p27^{Kip1}$ was almost completely inhibited in the presence of either compound, demonstrating that linichlorin A and gentian violet stabilize $p27^{Kip1}$ in HeLa cells after the protein translation. In fact, as expected, no significant change in the $p27^{Kip1}$ mRNA expression level was observed in the compound treated cells (Fig. 3c). In addition, the expression level of Skp2 protein was not affected under these conditions, although it was decreased in the presence of a higher concentration of gentian violet (Fig. S1).

Linichlorin A and gentian violet inhibit cell growth and delay cell cycle progression. Next, we examined the effect of these compounds on the growth of tumor and non-tumor cells. HeLa (human tumor cells), tsFT210 (mouse tumor cells) and NIH3T3 (mouse immortalized cells) were exposed to increasing concentrations of linichlorin A and gentian violet for 48 h and their viability was measured by WST-8 assay (Fig. 4a). The IC_{50} values for linichlorin A in HeLa, tsFT210 and NIH3T3 cells were 3.2, 1.6 and 12.7 μM, respectively, and 0.4, 0.6 and 5.3 μM for gentian violet, respectively. This shows that the compounds inhibited growth to a greater extent in HeLa and tsFT210 cells compared with NIH3T3 cells. When we compared the expression level of $p27^{Kip1}$ between two mouse cells (tsFT210 cells and NIH3T3 cells) by immunoblotting, the endogenous expression level of $p27^{Kip1}$ in growing cells was higher in NIH3T3 cells (Fig. 4b). However, in the range of the compound concentration examined, $p27^{Kip1}$ expression level was increased by the compounds dose-dependently only in tsFT210 cells, as it is in HeLa cells (Fig. 4b). Although the mechanism of this difference in the drug sensitivity remains to be elucidated, this difference may explain the stronger growth inhibitory effect of these compounds in cancer cell lines.

It is predicted that the G_1–S transition will be retarded when $p27^{Kip1}$ is stabilized. To confirm this, we examined the compounds' effects on the cell cycle progression using tsFT210 cells that have temperature sensitive *Cdc2* mutation. After tsFT210 cells were synchronized at G_2 phase by incubation at 39°C (0 h), the cells were treated with compounds at their respective IC_{50} values (linichlorin A 1.6 μM and gentian violet 0.6 μM) prior to incubation at 32°C for 8 and 12 h. As a

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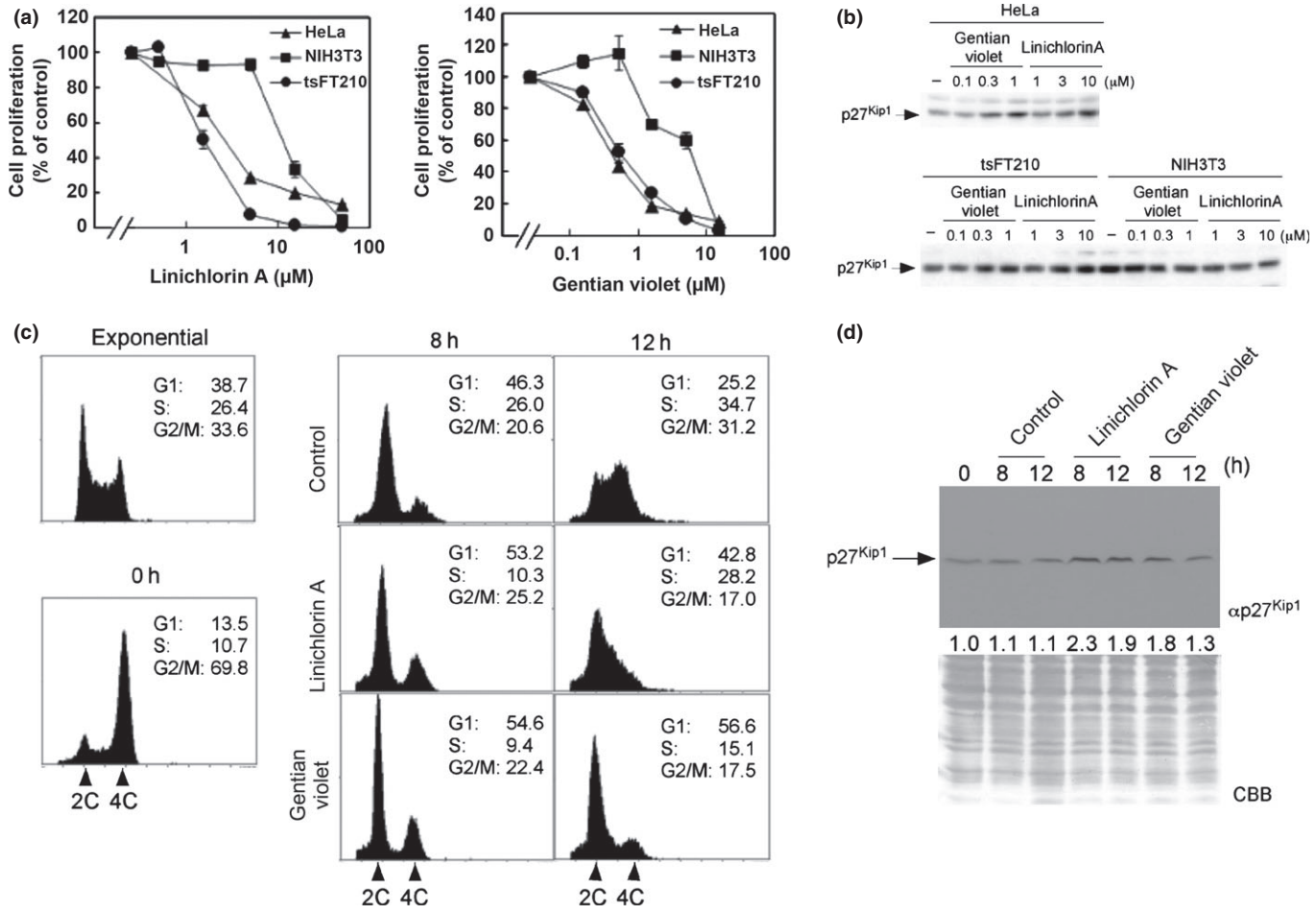

Fig. 4. Compounds induce cell growth and delay cell cycle progression. (a) Effect of linichlorin A and gentian violet on HeLa, tsFT210 and NIH3T3 cell growth by WST-8 assay at the indicated concentrations for 48 h. Data are representative of three replicates. (b) Dose-dependent effects of linichlorin A and gentian violet on the expression level of $p27^{Kip1}$ in HeLa, tsFT210 and NIH3T3 cells. Cells were treated with compounds at indicated concentrations for 18 h. Total proteins (20 μg each) from these cells were loaded on SDS-PAGE and processed for the detection of $p27^{Kip1}$ by immunoblotting. (c) Effect of compounds on cell cycle progression in tsFT210 cells. Cells were synchronized at G_2 phase at 39°C for 18 h, and the compounds were added at their respective IC_{50} values (linichlorin A 1.6 μM; gentian violet 0.6 μM) before the synchronized cells were released at 32°C at 0 h. Cells were harvested at the indicated times and stained with propidium iodide prior to flow cytometric analysis. DNA contents of G_1 (2C), S (2C–4C) and G_2/M (4C) cells are indicated by triangles. Analyses were repeated at least twice and the quantitative data in each panel represents the percentages of each phase. (d) Effect of compounds on $p27^{Kip1}$. tsFT210 cells treated with compounds as in b were harvested at the indicated times and the endogenous $p27^{Kip1}$ levels were examined by western blot with anti-$p27^{Kip1}$. The intensity of the bands was quantified using Image-J software (Wayne Rasband, Bethesda, MD, USA) and is indicated in multiple of that of 0 h control. CBB stain is also shown in the lower panel of the immunoblot.

result, the compounds delayed the initiation of S phase (Fig. 4c) and the levels of $p27^{Kip1}$ were significantly increased in the compound-treated cells (Fig. 4d).

Discussion

Low levels of $p27^{Kip1}$ and overexpression of Skp2 in many human carcinomas and lymphomas are caused primarily by the increased proteolysis of $p27^{Kip1}$ through ubiquitination by SCF^{Skp2} E3 ligase.[43–46] Thus, discovery of small molecule inhibitors through the development of screening systems that specifically target SCF^{Skp2} E3 ligase by blocking the interaction between the F-box protein (Skp2) and its substrate ($p27^{Kip1}$) presents an ideal cancer therapeutic.

We developed an HTS system for inhibitors of the interaction of Skp2–Cks1 and $p27^{Kip1}$. Through HTS and further study, we identified linichlorin A and gentian violet as the most potent compounds that inhibited the ubiquitin-proteasome dependent degradation of $p27^{Kip1}$ (Fig. 1d). Linichlorin A is a sesquiterpene lactone that was first isolated from *Centaurea linifolia* Vahl[47] and does not have any reported biological activity, whereas gentian violet (also known as crystal violet) is a triphenylmethane-classed dye that has antifungal, antibacterial and antiparasitic activities.[48] While both compounds have inhibitory effects on the binding between mAGSkp2–Cks1 and $p27^{Kip1}$ phosphopeptides, these compounds did not show any effect on the phosphorylation-dependent binding of other F-box proteins, β-TrCP1 and β-TrCP2. In addition, these compounds also did not show any effect on other phosphorylation dependent protein–protein interactions of PBD,[40] Pin1, 14-3-3 and Mdc1 (Watanabe N and Osada H, unpublished results). Therefore, we consider that the inhibitory effect of these compounds is specific to Skp2–$p27^{Kip1}$ interaction.

From the structure of Skp2 and Cks1 complexed with $p27^{Kip1}$ phosphopeptides,[38] it is predicted that these compounds bind to the pocket created by Skp2 and Cks1 rather than binding to the phosphorylated peptides. In fact, no inhibition on binding was observed in our screening system when the compounds were pre-mixed with phosphorylated peptides in 96-well plates and washed before the addition of Skp2–Cks1 containing lysates,

indicating that the compounds do not bind to the phosphopeptides but to the Skp2–Cks1 complex, as predicted. In this regard, the increase in the expression level of two other known Skp2–Cks1 target proteins, $p21^{Cip1}$[49] and p130,[50] by these compounds supports this idea (Fig. S2).

According to earlier findings,[13,14,17,18,20] the ubiquitination of $p27^{Kip1}$ is triggered by its phosphorylation by CDK2/cyclin E, followed by recognition of phosphorylated $p27^{Kip1}$ by SCF^{Skp2} E3 ligase and an adapter protein, Cks1, which polyubiquitinates $p27^{Kip1}$ and targets it for proteasomal degradation. Here, we found that linichlorin A and gentian violet had actually inhibited $p27^{Kip1}$ ubiquitination *in vitro* (Fig. 2c), as predicted by their Skp2–$p27^{Kip1}$ binding inhibitory activities, followed by stabilization of $p27^{Kip1}$ level in the compound-treated HeLa cells (Fig. 3a,b).

We have also demonstrated that linichlorin A and gentian violet have substantial, selective antiproliferative activity against cancer and transformed cells in the micromolar range (Fig. 4a). In many types of tumor cells, it is reported that Skp2 is overexpressed and the level of $p27^{Kip1}$ is downregulated. This downregulation of $p27^{Kip1}$ is considered to be responsible for the accelerated growth of tumor cells. Thus, the inhibition on Skp2-dependent degradation of $p27^{Kip1}$ may exhibit larger effects on the growth of tumor cells.

Although further analyses using cells with reduced or overexpressed levels of $p27^{Kip1}$ and/or Skp2 are necessary, our results strongly suggest that these compounds inhibit the growth of cancer cells at G1 phase of the cell cycle by stabilizing $p27^{Kip1}$ through the inhibition of the interaction between Skp2–Cks1 and $p27^{Kip1}$. Taken together, our study demonstrates a potential strategy for restoring $p27^{Kip1}$ levels in cancers using small molecule inhibitors.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Dose-dependent effect of linichlorin A and gentian violet on the expression level of SKP2 in HeLa cells.

Fig. S2. Effect of compounds on the level of $p21^{Cip1}$ and p130.