Zinc-finger protein 143 (ZNF143) is a transcription factor that has been identified as a human homolog of Xenopus Staf.\(^1\) In humans, ZNF143 has been suggested to play roles in various activities.\(^2\) Previous studies demonstrated that ZNF143 is involved in the cell cycle, cell viability, and drug resistance.\(^3\) Furthermore, the knockdown of ZNF143 expression by small interfering RNA (siRNA) downregulated the expression of various cell cycle/DNA replication-associated genes and inhibited the progression of the cell cycle and tumor cell growth.\(^6\) ZNF143 was also more strongly expressed in solid tumors than in adjacent non-tumor samples.\(^5\) In clinical specimens, ZNF143 protein levels have been correlated with clinical outcomes in lung adenocarcinoma.\(^5\) Therefore, ZNF143 is considered to be a promising and unique cancer drug target. However, molecules that inhibit the activity of ZNF143 have not yet been identified. We herein identified a novel small molecule N-(5-bromo-2-methoxyphenyl)-3-(pyridine-3-yl) propiolamide (YPC-21661) that inhibited ZNF143 promoter activity and down-regulated the expression of ZNF143-regulated genes, RAD51, PLK1, and Survivin, by inhibiting the binding of ZNF143 to DNA. In addition, YPC-21661 was cytotoxic and induced apoptosis in the human colon cancer cell line, HCT116 and human prostate cancer cell line, PC-3. 2-(pyridine-3-yl)ethynyl)-5-(2-(trifluoromethoxy)phenyl)-1,3,4-oxadiazole (YPC-22026), a metabolically stable derivative of YPC-21661, induced tumor regression accompanied by the suppression of ZNF143-regulated genes in a mouse xenograft model. The present study revealed that the inhibition of ZNF143 activity by small molecules induced tumor regression in vitro and in vivo; therefore, ZNF143 is a promising target of cancer therapeutics.

Materials and Methods

**Reagents.** YPC-21661 and YPC-22026 (Fig. 1) were chemically synthesized by Yakult Honsha (Tokyo, Japan). In *in vitro* assays, these drugs were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in all treatments was adjusted to 0.1%. In the *in vivo* study, YPC-22026 was dissolved in 5% glucose containing 10% (w/v) Tween80 and 5% (w/v) propylene glycol.

**Cell cultures.** The human colon cancer cell lines HCT116 and HT-29 were purchased from the American Type Culture Collection (Manassas, VA, USA). The human colon cancer cell line DLD-1, human non-small cell lung cancer cell line PC-9, and human endometrial adenocarcinoma cell line Ishikawa were purchased from DS Pharma Biomedical (Osaka, Japan). The human prostate cancer cell line, PC-3 and PC-3 cells stably expressing 3xFlag-tagged ZNF143 (PC-3/3xFlag-ZNF143) were obtained from the University of Occupational and Environmental Health, Japan.\(^5\) All cell lines were cultured in RPMI medium 1640 containing 10% (v/v) fetal bovine serum and maintained at 37°C in a 5% CO\(_2\) atmosphere.

**Plasmid construct.** In order to prepare the tandem repeat sequence of the Staf-binding site, human genomic DNA was amplified by PCR with the following primer pair: 5'-ACGC GTACTACGCTCCTCCACGCCTTTGCGCGGC-3' and 5'- CGGCCACTACGCTTCCACGCGTCTTTGCGCGGC-3'. Restriction enzyme sites are underlined. This PCR product was ligated into the MluI/Xhol site of the pGL3-basic vector (Promega, Madison, WI, USA). This plasmid was named pGL3-SBSx2-Luc. The pGL3 control vector, which contains the SV40 promoter upstream of the luciferase gene, was purchased from Promega.

**Reporter assays.** PC-3 cells that were stably transfected with the pGL3-SBSx2-Luc or pGL3 control vector were seeded on 96-well white plates. YPC-21661 or YPC-22026 was added to each well the next day. After a 16-h incubation, luciferase activity was detected using the Bright-Glo luciferase assay.

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Key words

anticancer drug, apoptosis, cell cycle, transcription factor, ZNF143
GAT-3

endogenous control. The primer sequences used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. The primer sequences used were as follows:

Quantitative RT-PCR. Total RNA was isolated from cancer cells or resected xenograft tumors using an RNAspin mini kit (GE Healthcare Biosciences, Piscataway, NJ, USA), according to the manufacturer’s protocol. First-strand cDNA was synthesized from isolated total RNA using the GoScript Reverse Transcription System (Promega). Quantitative RT-PCR was performed using the 7500 Fast real-time PCR system (Thermo Fisher Scientific). The results obtained were normalized for a viable cell count measured by the MTT assay.

Western blotting. Cancer cells were lysed in solubilization buffer (10 mM Tris-HCl, pH 7.4, 0.1% [w/v] Nonidet P-40, 0.1% [w/v] sodium deoxycholate, 0.1% [w/v] SDS, 150 mM NaCl, 1 mM EDTA, and 10 μg/mL aprotinin). Lysates were subjected to SDS-PAGE using 10% (w/v) gels under reducing conditions. The separated proteins were electrotransferred to polyvinylidene difluoride membranes using a semi-dry blotter. Membranes were treated with 5% (w/v) skimmed milk in 10 mM Tris-HCl, pH 7.4, 0.1% (v/v) Tween 20, and 150 mM NaCl for 1 h. Each membrane was then reacted with an anti-Survivin antibody (AF-886 R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s protocol. Briefly, exponentially growing cells were seeded at a density of 5 × 10^6 cells/well. Serially diluted YPC-21661 or YPC-22026 were added to each well the next day. After a 96-h incubation, the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt, WST-8, was added to each well. The attached cells were fixed and stained with propidium iodide using a cell cycle analysis kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s protocol. Cell viability was assessed in a 96-well plate using TetraCoral ONE (Kishida chemical, Osaka, Japan), according to the manufacturer’s protocol. Briefly, exponentially growing cells were seeded at a density of 5 × 10^6 cells/well. Serially diluted YPC-21661 or YPC-22026 were added to each well the next day. After a 96-h incubation, the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt, WST-8, was added to each well and the plates were incubated for 1 h at 37°C. Absorbance was then measured at 450 nm using a SPECTRA Max PLUS384 (Molecular Devices, Sunnyvale, CA, USA).

Evaluation of nuclear morphology. Cells were seeded in a 24-well plate and treated with YPC-21661. After a 24-h incubation, cells were stained in 0.5 μg/mL Hoechst 33342 in culture medium for 30 min. Nuclear morphology was visualized using a fluorescence microscope (Biozero BZ-8100, Keyence, Osaka, Japan).

DNA fragmentation assays. DNA fragmentation, which is characteristic of apoptosis, was quantitatively evaluated using Cell Death Detection ELISA PLUS (Roche Life Science, Indianapolis, IN, USA), according to the manual instructions. Briefly, the cytoplasmic fractions of untreated control and YPC-21661-treated cells were transferred onto a streptavidin-coated plate and incubated at room temperature for 2 h with a mixture of peroxidase-conjugated anti-DNA and biotin-labeled anti-histone. The plate was washed thoroughly and incubated with ABTS solution. Absorbance was then measured at 405 nm with a reference wavelength of 490 nm.

Analysis of cell cycle distribution. HCT116 cells were treated with or without YPC-21661 or YPC-22026. The attached cells were fixed and stained with propidium iodide using a cell cycle phase determination kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manual instructions. Cell cycle distribution was analyzed by flow cytometry using a GUAVA EasyCyte Plus System (Merck Millipore, Darmstadt, Germany).

Metabolic stability in mouse microsomes. BALB/c mouse hepatic microsomes were purchased from Xenotech, LLC (Kansas City, KS, USA). A total of 0.5 mL of the reaction mixture,
YPC-21661 inhibits ZNF143 transcriptional activity and down-regulates the expression of ZNF143 target genes. We first evaluated the effects of YPC-21661 on the transcriptional activity of ZNF143 using a luciferase reporter gene assay. We found that YPC-21661 inhibited ZNF143 promoter activity, but did not significantly inhibit SV40 promoter activity at concentrations up to 3.0 μmol/L (Fig. 2). ZNF143 plays important roles in the expression of DNA repair genes, cell cycle-related genes, and anti-apoptotic genes. Therefore, we examined the effects of YPC-21661 on the expression of these genes using real-time PCR and Western blotting. As shown in Figure 3, YPC-21661 decreased the expression of ZNF143 target gene mRNAs (RAD51, PLK1 and Survivin) (Fig. 3a) and their respective proteins (Fig. 3b). Collectively, these results show that YPC-21661 inhibits ZNF143 activity and down-regulates the expression of its target genes.

**Statistical analysis.** Statistical analyses were performed using Dunnett’s test. *P*-values <0.05 were considered significant.

**Results**

YPC-21661 inhibits ZNF143 transcriptional activity and down-regulates the expression of ZNF143 target genes. We first evaluated the effects of YPC-21661 on the transcriptional activity of ZNF143 using a luciferase reporter gene assay. We found that YPC-21661 inhibited ZNF143 promoter activity, but did not significantly inhibit SV40 promoter activity at concentrations up to 3.0 μmol/L (Fig. 2). ZNF143 plays important roles in the expression of DNA repair genes, cell cycle-related genes, and anti-apoptotic genes. Therefore, we examined the effects of YPC-21661 on the expression of these genes using real-time PCR and Western blotting. As shown in Figure 3, YPC-21661 decreased the expression of ZNF143 target gene mRNAs (RAD51, PLK1 and Survivin) (Fig. 3a) and their respective proteins (Fig. 3b). Collectively, these results show that YPC-21661 inhibits ZNF143 activity and down-regulates the expression of its target genes.

**YPC-21661 inhibits the DNA binding of ZNF143.** In order to investigate the effects of YPC-21661 on ZNF143 binding at...
the STAF binding site in the promoter region of ZNF143 target genes, ChIP assays were performed after PC-3/3xFlag-ZNF143 was treated with YPC-21661 for 24 h. As shown in Figure 4, YPC-21661 decreased the binding of ZNF143 to the STAF binding site in a dose-dependent manner (Fig. 4a,b).

**YPC-21661 is cytotoxic to tumor cells and inhibits cell cycle progression by inducing apoptosis.** The cytotoxic effects of YPC-21661 were examined in HCT116 and PC-3 cells. YPC-21661 was potently cytotoxic to HCT116 and PC-3 cells with 50% inhibitory concentration (IC50) values of 6.0 and 6.8 nmol/L, respectively (Fig. 5a). We then analyzed the effects of YPC-21661 on cell cycle distribution because ZNF143 regulates the expression of cell cycle-related genes.\(^{(3,7,8)}\) Histograms were shown in Figure 5(b). Increases in the numbers of G2/M phase cells and subG1 cells were observed when HCT116 cells were treated with YPC-21661 at 0.1 µmol/L (Fig. 5b). Increases in the numbers of subG1 cells suggest that YPC-21661 induces apoptosis; therefore, we investigated whether the cytotoxic effects of YPC-21661 are caused by apoptosis. PC-3 and HCT116 cells were treated with YPC-21661 at 0.1 µmol/L (Fig. 5b). Increases in the numbers of subG1 cells suggest that YPC-21661 induces apoptosis; therefore, we investigated whether the cytotoxic effects of YPC-21661 are caused by apoptosis. PC-3 and HCT116 cells were treated with YPC-21661 and stained with Hoechst 33342. Condensed and fragmented nuclei were observed under a fluorescent microscope. (b) HCT116 cells were treated with YPC-21661 at the indicated concentrations for 24 h. Fragmented DNA was quantified, according to the manual instructions. The amount of fragmented DNA was expressed as a relative value (% of control). Data are the means ± SD of triplicate determinations. (c) Western blotting for PARP and cleaved PARP expressed in HCT116 cells treated with YPC-21661 for 16 h at the indicated concentrations.
YPC-21661 and YPC-22026 inhibit ZNF143 activity

YPC-21661 and YPC-22026 inhibit ZNF143 activity in vitro and in vivo. (a) PC-3 cells stably transfected with the pGL3-SBSx2-Luc (black bars) or pGL3 control vector (white bars) were treated with YPC-22026 at the indicated concentrations. After 16 h, luciferase activity was measured. Data are the means ± SD of triplicate determinations. (b) PC-3 (open circles) and HCT116 (open triangles) cells were treated with YPC-22026 for 96 h. Cell viability was assessed by WST-8 and presented as a percentage of the value for the control culture. (c) BALB/c nude mice (n = 5/group) were inoculated subcutaneously with HCT116 cells. After tumors had formed, mice were treated intraperitoneally with 50 (open triangles) or 100 (open squares) mg/kg YPC-22026 or with vehicle only (open circles). Data are expressed as the mean ± SD of triplicate determinations. (b) PC-3 (open circles) and HCT116 (open triangles) cells were treated with YPC-21661 and YPC-22026 at the indicated concentrations. After 16 h, luciferase activity was measured. Data are the means ± SD of triplicate determinations. (b) PC-3 cells stably transfected with the pGL3-SBSx2-Luc (black bars) or pGL3 control vector (white bars) were treated with YPC-22026 at the indicated concentrations. After 16 h, luciferase activity was measured. Data are the means ± SD of triplicate determinations.

Effects of YPC-22026 on tumor growth and ZNF143 activity

In the in vivo experiments, the treatment with YPC-21661 led to the accumulation of fragmented DNA at 0.3 μmol/L (Fig. 6b) and induced PARP cleavage in a dose-dependent manner (Fig. 6c).

These results demonstrate that YPC-21661 is cytotoxic to tumor cells by inducing G2/M phase cell cycle arrest and apoptosis.

YPC-22026 inhibits the growth of human xenograft tumors by inhibiting the expression of ZNF143-regulated genes

Since YPC-21661 is unstable in mouse liver microsomes, we synthesized YPC-22026 in order to improve stability. The in vitro activity of YPC-22026 was evaluated using a luciferase reporter assay and MTT assay. YPC-22026 inhibited ZNF143 activity with an IC50 value of 9.0 μmol/L (Fig. 7a), and was cytotoxic to HCT116 and PC-3 with IC50 values of 0.33 and 0.66 μmol/L, respectively (Fig. 7b). In addition, YPC-22026 induced G2/M arrest (Fig. S1A) and decreased the binding of ZNF143 to the STAF binding site (Fig. S1B). The in vitro activity of YPC-22026 was weaker than that of YPC-21661, whereas YPC-22026 exhibited cytotoxicity by inhibiting ZNF143 activity. Therefore, we evaluated the in vivo antitumor activity of YPC-22026. In the HCT116 xenograft model, the treatment with YPC-22026 at 50 or 100 mg/kg significantly inhibited the growth of tumors, with IR values of 40.8% and 56.1%, respectively, on day 22 (Fig. 7c). In order to confirm whether YPC-22026 alters ZNF143 activity in tumors during the drug treatment, we evaluated the effects of YPC-22026 on the intratumoral expression of the ZNF143 target genes, RAD51, PLK1, and Survivin. Mice with xenografted PC-3 tumors received a 3-day continuous infusion of YPC-22026 at 75 mg/kg. Animals treated with YPC-22026 showed a significant decrease in the intratumoral expression levels of all ZNF143 target genes analyzed after 8 and 24 h from the last administration (Fig. 7d). These results show that YPC-22026 inhibits the growth of tumors by decreasing the intratumoral expression levels of ZNF143 target genes.

Discussion

YPC-21661 is a novel ZNF143 inhibitor that has been identified using a luciferase reporter assay. YPC-21661 downregulated the expression of ZNF143 target genes by inhibiting the binding of ZNF143 to DNA. In addition, YPC-21661 was cytotoxic to cancer cells through its induction of G2/M phase cell cycle arrest and apoptosis. These results were consistent with previous findings obtained from ZNF143 knockdown experiments. This agreement confirmed that YPC-21661 inhibits ZNF143 activity. In addition, the ZNF143-over-expressing cell line, PC-3/3xFlag-ZNF143, was more vulnerable to YPC-21661 and YPC-22026 than parent cells (Fig. S2A,B), suggesting that the efficacy of YPC compounds depend on ZNF143 expression levels. These results also indicate that YPC compounds are potent inhibitors of ZNF143. On the other hand, the efficacy of YPC compounds on cancer cell lines that intrinsically express ZNF143 was not related to ZNF143 expression levels (Fig. S3 and Table S1). This discordance may be attributed to differences in genetic backgrounds; PC-3 and PC-3/3xFlag-ZNF143 have similar genetic backgrounds; therefore, the efficacy of YPC compounds is relevant to ZNF143 expression levels. However, the cancer cells used in the present study have various genetic backgrounds, and their growth is not decided based only on the expression levels of ZNF143; therefore, the efficacies of YPC compounds are not relevant to ZNF143 expression levels. YPC-21661-induced apoptosis may be caused by the down-regulation of the anti-apoptotic factor Survivin (Fig. 3b). However, previous studies...

For cleaved poly (ADP-ribose) polymerase (PARP). As shown in Figure 6(b, c), the treatment with YPC-21661 led to the accumulation of fragmented DNA at 0.3 μmol/L. (Fig. 6b) and induced PARP cleavage in a dose-dependent manner (Fig. 6c). These results demonstrate that YPC-21661 is cytotoxic to tumor cells by inducing G2/M phase cell cycle arrest and apoptosis.

YPC-22026 inhibits the growth of human xenograft tumors by inhibiting the expression of ZNF143-regulated genes. Since YPC-21661 is unstable in mouse liver microsomes, we synthesized YPC-22026 in order to improve stability. The in vitro activity of YPC-22026 was evaluated using a luciferase reporter assay and MTT assay. YPC-22026 inhibited ZNF143 activity with an IC50 value of 9.0 μmol/L (Fig. 7a), and was cytotoxic to HCT116 and PC-3 with IC50 values of 0.33 and 0.66 μmol/L, respectively (Fig. 7b). In addition, YPC-22026 induced G2/M arrest (Fig. S1A) and decreased the binding of ZNF143 to the STAF binding site (Fig. S1B). The in vitro activity of YPC-22026 was weaker than that of YPC-21661, whereas YPC-22026 exhibited cytotoxicity by inhibiting ZNF143 activity. Therefore, we evaluated the in vivo antitumor activity of YPC-22026. In the HCT116 xenograft model, the treatment with YPC-22026 at 50 or 100 mg/kg significantly inhibited the growth of tumors, with IR values of 40.8% and 56.1%, respectively, on day 22 (Fig. 7c). In order to confirm whether YPC-22026 alters ZNF143 activity in tumors during the drug treatment, we evaluated the effects of YPC-22026 on the intratumoral expression of the ZNF143 target genes, RAD51, PLK1, and Survivin. Mice with xenografted PC-3 tumors received a 3-day continuous infusion of YPC-22026 at 75 mg/kg. Animals treated with YPC-22026 showed a significant decrease in the intratumoral expression levels of all ZNF143 target genes analyzed after 8 and 24 h from the last administration (Fig. 7d). These results show that YPC-22026 inhibits the growth of tumors by decreasing the intratumoral expression levels of ZNF143 target genes.

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reported that the down-regulation of Survivin reduces the number of G2/M phase cells.\(^{13,14}\) Therefore, G2/M arrest induced by YPC-21661 does not appear to be essential to apoptosis. YPC-22026, designed to improve stability in human liver microsomes, exhibited anti-tumor activity in the HCT116 xenograft model by inhibiting the expression of ZNF143 target genes. This \textit{in vivo} anti-tumor activity suggests that ZNF143 inhibitors are promising anti-cancer drugs.

ZNF143 is a transcription factor related to the cell cycle. E2F and c-Myc are well-known transcription factors that are involved in cell cycle progression and cancer malignancy. Small molecules that inhibit E2F or c-Myc transcriptional activity induce cell cycle arrest at the G0/G1 phase.\(^{15,20}\) On the other hand, the inhibition of ZNF143 by YPC-21661 induced G2/M arrest (Fig. 5b) in accordance with the findings of a previous knockdown analysis.\(^{3}\) These results indicate that the role of ZNF143 in cancer cell proliferation is distinct from those of E2F and c-Myc.

In the \textit{in vivo} study, the amide bond included in YPC-21661 is metabolically unstable. Therefore, we converted the amide bond for 1,3,4-oxadiazole, which is a biosostere of the amide bond, in order to improve the elimination half-life of YPC-22026 in mouse microsomes over that of YPC-21661 (34.4 and <1.7 min, respectively). ZNF143 controls the expression of various genes related to cell survival.\(^{3,7,10}\) Therefore, we anticipated that ZNF143 inhibitors exhibit severe toxicity against mice. However, the toxicity of YPC-22026 against mice only led to slight weight loss (<20%) at therapeutically effective doses (data not shown).

The expression of ZNF143 is activated by DNA damage reagents such as etoposide, cisplatin, and adriamycin.\(^{6}\) In addition, ZNF143 binds to cisplatin-modified DNA and is involved in cisplatin resistance;\(^{14,6}\) therefore, YPC-21661 and 22026 may augment the efficacy of cisplatin. Further extensive studies on this matter are needed.

In the present study, we demonstrated that YPC-21661 inhibited the activity of ZNF143; however, the mechanisms responsible for its inhibitory effects have not yet been elucidated. Therefore, further studies are needed in order to develop YPC-22026 or its derivatives as novel anti-cancer drugs. In conclusion, YPC-21661 and YPC-22026 appear to be the first ZNF143 inhibitors to exhibit anti-cancer activities \textit{in vitro} and \textit{in vivo}. These results indicate that YPC-21661 and 22026 are promising first-in-class drug seeds.

**Disclosure Statement**

Hirotaka Haibara, Ryuta Yamazaki, Yukiko Nishiyama, Masa-hiro Ono, Tsuneyuki Kobayashi, Atsuko Hokkyo-Itagaki, Fukiko Nishisaka, Hiroyuki Nishiyama, Akinobu Kurita, and Takeshi Matsuizaki are employees of Yakult Honsha Co., Ltd., Tokyo, Japan.

**Supporting Information**

Additional Supporting Information may be found online in the supporting information tab for this article.
Fig. S1. Effects of YPC-22026 on the cell cycle and DNA binding of ZNF143.

Fig. S2. Effects of YPC-21661 and YPC-22026 on the cell viability of PC-3/3xFlag-ZNF143.

Fig. S3. Analysis of ZNF143 expression in cancer cells.

Table S1. IC$_{50}$ values by the 96-h treatment of YPC-21661 and YPC-22026 in human cancer cell lines.