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Hypoxia-Selective Growth Inhibition of Cancer Cells by Furospinosulin-1, a Furanosesterterpene Isolated from an Indonesian Marine Sponge

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It is generally accepted that cancer cells, which have adapted to the hypoxic environments in tumor tissues, aggravate cancer pathology by promoting tumor growth, angiogenesis, metastasis, and drug resistance. Therefore, compounds that selectively inhibit the growth of tumor cells in hypoxic environments are expected to provide new leads for promising anticancer drugs. Furospinosulin-1, a marine-sponge-derived furanosesterterpene, exhibited selective antiproliferative activity against DU145 human prostate cancer cells under hypoxic conditions in concentrations ranging from 1 to 100 μM. Furospinosulin-1 also demonstrated antitumor activity at 10–50 mg kg^{-1} oral administration in a mouse model inoculated with sarcoma S180 cells. Mechanistic analysis revealed that furospinosulin-1 suppresses transcription of the insulin-like growth factor-2 gene (*IGF-2*), which is selectively induced under hypoxic conditions through prevention of the binding of nuclear proteins to the Sp1 consensus sequence in the *IGF-2* promoter region.

Introduction

The hypoxic nature of tumor environments is now recognized as an important factor for tumor growth, angiogenesis, metastasis, and response to chemotherapy and irradiation. Tumor cells in hypoxic environments exhibit resistance to chemotherapy and irradiation and promote angiogenesis.[1] In addition, the hypoxic environment of a tumor is unlike that in normal tissues. Therefore, compounds that selectively inhibit growth of tumor cells in hypoxic environments are expected to provide promising new leads for anticancer drugs.

Regulation systems, such as hypoxia-inducible factor (HIF-1), the kinase mammalian target of rapamycin (mTOR), and the unfolded protein response (UPR), are involved in the adaptation of cancer cells to hypoxia.[2] Among these, the heterodimeric transcription factor of HIF-1, which comprises an oxygen-regulated α-subunit and a constitutively expressed β-subunit, has been studied extensively as a mediator of the cellular response to hypoxia. In addition, decreased HIF-1α activity is usually associated with a slower growing and less angiogenic tumor phenotype.[3] Furthermore, a number of compounds, such as echinomycin,[4] chetomin,[5] manassantin B,[6] laurenditerpenol,[7] strongylophorines,[8] and PX-478,[9] have been found to inhibit HIF-1α or its signaling pathway. Other regulation systems for adaptation of cancer cells in hypoxic environments may exist, as the expression of an estimated 1000 genes is altered under hypoxic conditions.[10,11]

The marine environment has rich chemical and biological diversity and, as such, has been a valuable source of drug lead compounds. Bioactive compounds from marine organisms are also useful tools for identifying novel drug targets. We have focused on the discovery of molecular-targeted anticancer natural products from marine organisms. This work has resulted in the discovery of several active compounds, including cortistatins, anti-angiogenic new steroidal alkaloids;[12] aaptamine, a benzonaphthyridine alkaloid activating p21 promoter;[13] and agosterol A, a polyhydroxylated sterol acetate that reverses multidrug resistance (MDR) of cancer cells. Agosterol A has been particularly useful for analysis of the drug transport function of multidrug resistance-associated protein 1 (MRP1).[14]

Following these findings, we established a new screening system to identify hypoxia-selective growth inhibitors with potential to become cancer drug leads that exhibit novel mechanisms of action. Using bioassay-guided separation, we isolated furospinosulin-1, a furanosesterterpene from the Indonesian marine sponge *Dactylospongia elegans*.[15] Furospinosulin-1 has been known to exhibit several biological activities, including antiproliferative activity against colon cancer HCT-116 cells (IC_{50} = 155 μM),[16] toxicity toward brine shrimp (IC_{50} = 10 μg mL^{-1}),[17] and inhibitory activity toward Cdc25A (IC_{50} = 2.5 μM, using an enzymatic assay).[18] In this study, we describe the hypoxia-selective growth inhibition and antitumor activity of furospinosulin-1 in detail. Furthermore, we have elucidated that insulin-like growth factor (IGF)-2 contributes strongly to the growth of cancer cells in hypoxic environments via mechanistic analysis of furospinosulin-1.

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Results

Hypoxia-selective growth inhibition of furospinosulin-1

In the course of our search for hypoxia-selective inhibitors of cancer cell growth, we isolated the furanosesterterpene furospinosulin-1 (Figure 1). Incubation of human prostate cancer

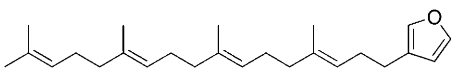

Figure 1. Chemical structure of furospinosulin-1.

DU145 cells for 24 h in the presence of furospinosulin-1 under normoxic or hypoxic conditions resulted in the selective inhibition of hypoxic cells in a dose-dependent manner at concentrations ranging from 1.0 to 100 μM. Maximal growth inhibition of 60% was achieved with 100 and 300 μM furospinosulin-1 under hypoxic conditions (Figure 2A). In contrast, the conventional antitumor drug cisplatin did not exhibit hypoxia-selective growth inhibition against DU145 cells (Figure 2B). Furospinosulin-1 also demonstrated hypoxia-selective inhibition against growth of human cervical cancer HeLa cells and mouse sarcoma S180 cells (Figure 2C and 2D).

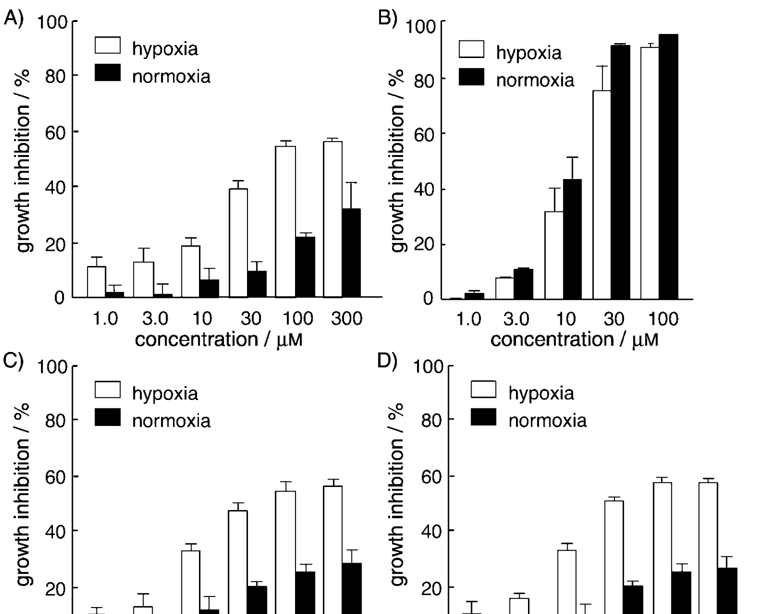

Figure 2. Hypoxia-selective growth inhibition of furospinosulin-1. DU145 cells (1×10^4 cells per well [200 μL]) were pre-incubated in a 96-well plate for 12 h under normoxic or hypoxic conditions. The cells were then treated with the indicated concentrations of furospinosulin-1 (A) or cisplatin (B) for 24 h under normoxic or hypoxic conditions. The growth inhibition rate was calculated as a percentage relative to negative controls. The effect of furospinosulin-1 on HeLa cells (C) and S180 cells (D) was also investigated. Data are shown as mean ±SD and represent the mean of four separate experiments performed in triplicate.

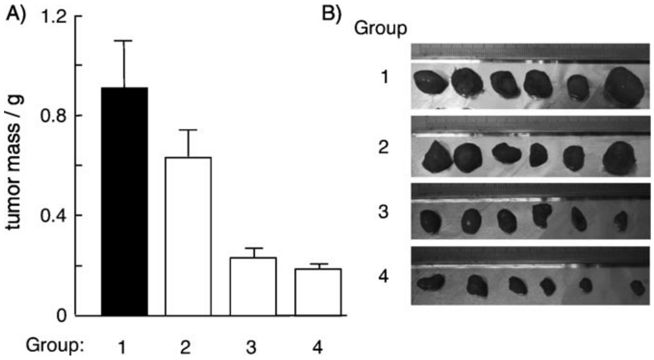

Figure 3. Antitumor effect of furospinosulin-1. S180 cells (1×10^6 cells per mouse) were inoculated subcutaneously into ddY mice. One week after inoculation, furospinosulin-1 was administrated orally (Group 1, control; Group 2, 10 mg kg^{-1}; Group 3, 25 mg kg^{-1}; and Group 4, 50 mg kg^{-1}) every other day for two weeks. The tumors were then isolated and weighed to calculate the ratio of inhibition. Data represent the tumor weight of each group (A) and images of the isolated tumors (B), and are presented as mean ±SD.

Antitumor effect of furospinosulin-1 in a sarcoma S180 mouse model

We next evaluated the antitumor effect of furospinosulin-1. A 10 mg kg^{-1} oral dose of furospinosulin-1 slightly (30%) suppressed tumor growth relative to the control; 74% and 79% decreases in tumor weight were observed with 25 and 50 mg kg^{-1} furospinosulin-1 administrations, respectively (Figure 3). Side effects, such as weight loss, diarrhea, and changes in behavior and feeding, were not observed during the experimental period.

Effects of furospinosulin-1 on the accumulation of HIF-1α and the production of VEGF

The effects of furospinosulin-1 on the accumulation of HIF-1α, an important transcription factor for adaptation of cancer cells to hypoxic environments, and the production of vascular endothelial cells growth factor (VEGF), which is regulated by HIF-1, were investigated by Western blotting analysis (Figure 4). DU145 cells showed an increased accumulation of HIF-1α and production of VEGF under hypoxic conditions. However, 30–100 μM concentrations of furospinosurin-1 did not decrease the levels of HIF-1α or VEGF in hypoxic DU145 cells. At a higher concentration (300 μM), furospinosulin-1 slightly inhibited HIF-1α accumulation and VEGF production. These results suggest that the hypoxia-selective growth inhibition of cancer cells by furospinosulin-1 is not derived from inhibition of HIF-1α function.

Effects of furospinosulin-1 on hypoxia-related genes

To elucidate the mechanism of action of furospinosulin-1, we analyzed the gene expression profile of cul-

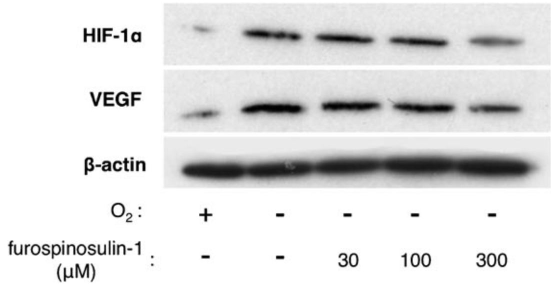

Figure 4. Effect of furospinosulin-1 on the accumulation of HIF-1α and production of VEGF. DU145 cells were incubated in six-well plates for 24 h under normoxic or hypoxic conditions in the presence or absence of the indicated concentrations of furospinosulin-1. VEGF in the culture medium was detected by immunoprecipitation with anti-VEGF antibody. To detect HIF-1α and β-actin, cell lysates were resolved by SDS-PAGE and incubated with anti-HIF-1α or anti-β-actin antibodies.

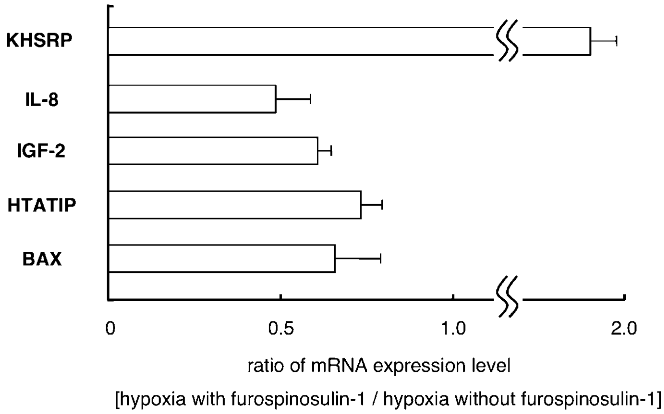

Figure 5. Effect of furospinosulin-1 on the expression of hypoxia-related genes. Following analysis of the gene expression profile of DU145 cells, the expression levels of each gene were quantified by the GEArray Expression Analysis Suite (SuperArray Bioscience) with the background subtracted. Data for genes whose expressions were altered by furospinosulin-1 treatment under hypoxic conditions were reported as a ratio of the expression levels under hypoxic conditions with and without furospinosulin-1.

tured DU145 cells under normoxic and hypoxic conditions in the presence or absence of furospinosulin-1 (30 μM), using a pathway-specific OligoGE array to detect 113 genes involved in the hypoxia-signaling pathway. As shown in Supporting Information table S1, 41 genes, comprised of 15 transcription factors and regulators, 12 metabolism (protein, lipid and nucleic acid)-related genes, four cell growth-related genes, four signal transduction-related genes, three apoptosis-related genes, and three stress-response genes, were all up-regulated under hypoxic conditions in comparison to those under normoxic conditions. Nine of the 113 genes, including six metabolism-related genes, two apoptosis-related genes, and one stress–response gene, were down-regulated under hypoxic conditions.

Among the 41 up-regulated genes, the expression levels of four in particular: interleukin-8 (*IL-8*), insulin-like growth factor-2 (*IGF-2*), the 60 kDa HIV-1 Tat-interacting protein (*HTATIP*), and Bcl2-associated X protein (*BAX*), were decreased following treatment with furospinosulin-1 for 12 h under hypoxic conditions. Additionally, further induction of KH-type splicing regulatory protein (*KHSRP*) was observed under hypoxic conditions after furospinosulin-1 treatment (Figure 5). On the other hand, expression of the nine genes that were down-regulated under hypoxic conditions were not affected by treatment with furospinosulin-1.

Furospinosulin-1 inhibits hypoxia-induced expression of IGF-2

We next evaluated the protein expression levels of five genes that were altered by furospinosulin-1 treatment under hypoxic conditions. Although the induction of *KHSRP* and *BAX* genes were suppressed by furospinosulin-1, the expression levels of both KHSRP and BAX proteins did not change following treatment (data not shown). In addition, HTATIP protein could not be detected in the DU145 cells due to the detection limit of chemiluminescence intensity. The expression level of IL-8 protein in the DU145 cell culture medium was slightly decreased by furospinosulin-1 treatment under both normoxic and hypoxic conditions; however, no selectivity was observed between these conditions. Moreover, the amount of IL-8 protein

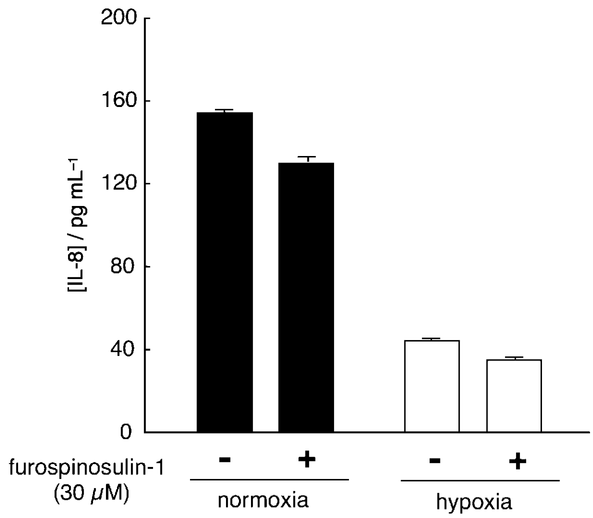

Figure 6. Production of IL-8 from DU145 cells cultured with furospinosulin-1 under normoxic or hypoxic conditions. DU145 cells were incubated in six-well plates for 12 h under normoxic or hypoxic conditions in the presence or absence of 30 μM furospinosulin-1. IL-8 in the culture medium was quantified using the human IL-8 ELISA kit, according to the manufacturer's instructions. Data are shown as mean ±SD and represent the mean of three separate experiments performed in triplicate.

under normoxic conditions was higher than that under hypoxic conditions, as shown in Figure 6.

In contrast, variation in the protein level of IGF-2 correlated with the gene expression profile of *IGF-2*. Indeed, IGF-2 protein expression was elevated under hypoxic conditions as compared with normoxic conditions. Treatment with furospinosulin-1 (30–300 μM) diminished this protein expression, as shown in Figure 7. Because IGF-2 protein enhances autophosphorylation of the IGF-1 receptor (IGF-1R) and insulin receptor (IR), which correspond to the IGF-2 protein receptors,[19] we examined the effect of furospinosulin-1 on the phosphorylation

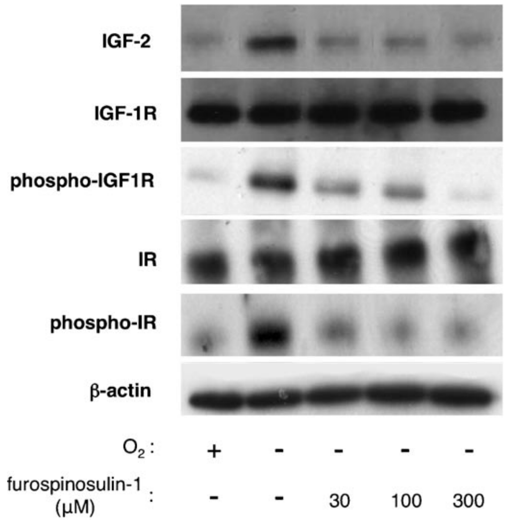

Figure 7. Inhibition of IGF-2 expression and autophosphorylation of the IGF-1 and insulin receptors by furospinosulin-1. DU145 cells were incubated in a six-well plate for 24 h under normoxic or hypoxic conditions in the presence or absence of the indicated concentrations of furospinosulin-1. IGF-2 in culture medium was then detected by immunoprecipitation with anti-IGF-2 antibody. Equal amounts of cell lysate were incubated with protein G agarose beads following treatment of anti-IGF-1R, anti-IR, or anti-β-actin antibodies. The immunoprecipitates were then resolved by 12% SDS-PAGE, transferred to PVDF membrane, and probed with appropriate antibodies.

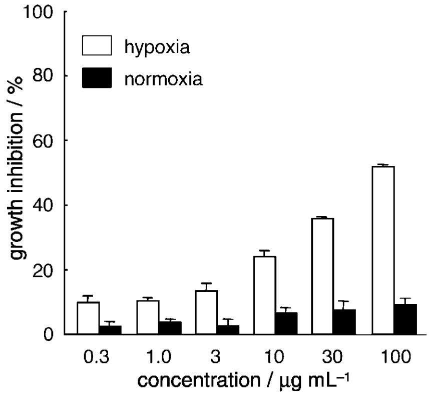

Figure 8. Effect of anti-IGF-2 antibody on the proliferation of DU145 cells under normoxic and hypoxic conditions. DU145 cells [1×10^4 cells per well (200 μL)] were pre-incubated in a 96-well plate for 12 h under normoxic or hypoxic conditions. Next, the cells were treated with the indicated concentrations of anti-IGF-2 antibody for 24 h under normoxic or hypoxic conditions in RPMI 1640 supplemented with 1% fetal bovine serum (FBS). The growth inhibition rate was calculated as a percentage with respect to the negative controls. Data are shown represent the mean ±SD of three separate experiments performed in triplicate.

levels of the IGF-1R and IR proteins. As expected, hypoxia-induced autophosphorylation of IGF-1R and IR proteins was suppressed by the furospinosulin-1 treatment. These findings indicated that furospinosulin-1 suppressed the IGF-2 protein expression that was selectively induced under hypoxic conditions, and that the mechanism of action of furospinosulin-1 may involve inhibition of *IGF-2* gene expression. We next examined whether IGF-2 protein deficiency induces an antiproliferative effect on DU145 cells under hypoxic conditions (Figure 8). When DU145 cells were incubated for 24 h in the presence of anti-IGF-2 antibody (0.3–100 μg mL^{-1}) under normoxic or hypoxic conditions, DU145 cellular proliferation was selectively inhibited under hypoxic conditions in a dose-dependent fashion. This indicates that suppression of IGF-2 protein expression by furospinosulin-1 strongly contributes to the hypoxia-specific inhibition of DU145 cell growth.

Effect of furospinosulin-1 on the promoter region of the *IGF-2* gene

It has been proposed that furospinosulin-1 selectively inhibits the transcription of *IGF-2* under hypoxic conditions. A recent study indicated that human hepatocarcinoma HepG2 cells in hypoxia showed increased expression of *IGF-2* mRNA.[20] Additionally, the P3 region of the *IGF-2* promoter, which contains the consensus sequences of EGR-1, Sp1, and HIF-1, is known to be important for transcription of *IGF-2* under hypoxic conditions.[20] Therefore, we examined the effect of furospinosulin-1 on the consensus sequences of EGR-1, Sp1, and HIF-1 by electrophoretic mobility shift assay (EMSA). As shown in Figure 9, the oligonucleotides containing the consensus sequences of EGR-1, Sp1, or HIF-1 formed a complex with nuclear proteins obtained from DU145 cells cultured under hypoxic conditions (Figure 9; lanes 2, 6, and 10). In contrast, nuclear proteins isolated from DU145 cells cultured under normoxic condition did not unite with these oligonucle-

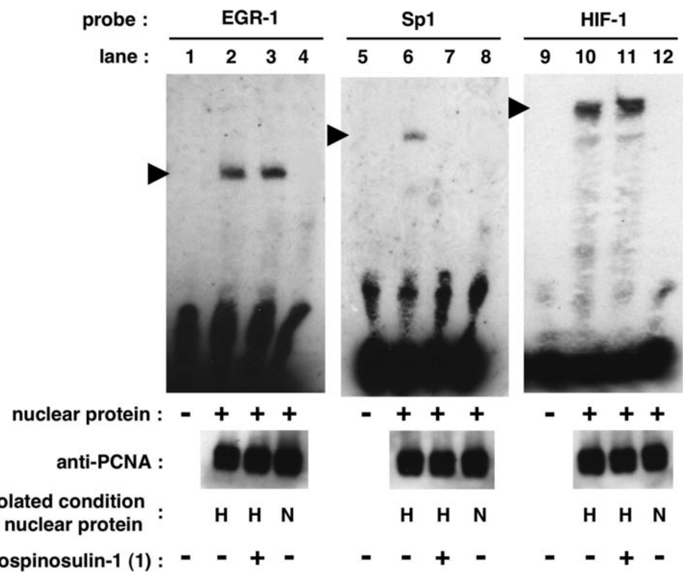

Figure 9. Inhibitory effect of furospinosulin-1 on complex formation of nuclear proteins with oligonucleotides containing consensus sequences of EGR-1, Sp1, and HIF-1. An electrophoretic mobility shift assay (EMSA) was performed using biotinylated double-stranded oligonucleotides containing the consensus sequence of EGR-1, Sp1, or HIF-1 with nuclear extracts derived from the DU145 cells under normoxic (**N**) or hypoxic conditions (**H**) as described in the Experimental Section. Concentration of the nuclear proteins was determined by an established BCA method and confirmed by Western blotting with anti-proliferating cell nuclear antigen (PCNA) antibody. Arrowheads indicate specific retarded and super-shifted bands.

otides (Figure 9; lanes 4, 8, and 12). On the other hand, furospinosulin-1 abrogated complex formation between nuclear proteins and the oligonucleotide containing the consensus sequence of Sp1, whereas this compound did not affect complex formation for those oligonucleotides containing the consensus sequences of EGR-1 or HIF-1α. These data indicate that furospinosulin-1 suppresses *IGF-2* transcription by inhibiting complex formation of nuclear proteins with the oligonucleotide containing the Sp1 consensus sequence.

Discussion

Recent studies have revealed that cancer cells adapt to hypoxic environments and aggravate cancer pathology. Therefore, we attempted to search for hypoxia-selective growth inhibitors for cancer cells from the metabolites of marine organisms. The furanosesterterpene furospinosulin-1 was isolated from an Indonesian marine sponge through bioassay-guided separation. Administration of furospinosulin-1 showed a significant antitumor effect in a mouse model inoculated with S180 sarcoma cells, with no side effects (Figure 3). Therefore, we expected that furospinosulin-1 will be a new lead for anticancer drugs that target hypoxia-adapted cancer cells. Mechanistic analysis of furospinosulin-1 strongly suggested that hypoxia-selective growth inhibition by furospinosulin-1 stems from inhibition of *IGF-2* transcription, a gene that is selectively induced under hypoxic conditions, through inhibition of the complex formation between nuclear proteins and the Sp1 consensus sequence in the *IGF-2* promoter region (Figure 7 and Figure 9).

The IGF system is comprised of two ligands (IGF-1 and IGF-2), six high affinity binding proteins (IGFBP 1–6), and three cell-surface receptors [IGF-1R, IGF-2 receptor (IGF-2R) and insulin receptor (IR)]. Among these, IGF-1R and IR are members of the tyrosine kinase family, while IGF-2R acts as a scavenger receptor for IGF-2. The IGF-1 ligand binds IGF-1R with high affinity, whereas the IGF-2 ligand binds to IGF-1R, IGF-2R, and IR with an affinity similar to that of insulin.[19] IGF-1R is frequently overexpressed in many types of cancer cells, mediates proliferation, and protects from apoptosis.[21] Therefore, IGF-1R has become a target for cancer chemotherapy. Although more than ten small molecules that inhibit IGF-1R kinase activity are in various stages of drug development, almost all of these inhibitors have problems with toxicity, due to the high degree of homology between IGF-1R and IR.[22] In contrast, the IGF-2 ligand is known to be a potent mitogen and is expressed in various human cancers.[23–26] However, the precise role of the IGF-2 ligand induced in cancer cells, particularly under hypoxic conditions, remains unknown. In our study, mechanistic analysis of furospinosulin-1 confirmed that this IGF-2 deficiency resulted in the hypoxia-selective inhibition of DU145 cell growth (Figure 8). As inhibition of IGF-2 expression is expected to have a selective antiproliferative effect on the hypoxia-adapted cancer cells that promote cancer pathology, furospinosulin-1, which inhibits IGF-2 expression, may be a more promising lead for anticancer drugs than current IGF-1R inhibitors.

The human *IGF-2* gene contains four promoters (P1–P4), which are expressed in a tissue-specific and development-dependent way.[27] Exposure of HepG2 cells to hypoxic conditions results in the overexpression of *IGF-2* mRNA, which is 6.0 kb in size and is transcribed under P3 promoter control.[20] The genome sequence of P3 indicated that this is a complex promoter with at least nine binding sites for transcription factors such as HIF-1, Sp1, and EGR-1.[28–30] As shown in Figure 9, the hypoxia-selective growth inhibition of DU145 cells by furospinosulin-1 results from inhibition of the binding of nuclear proteins to the consensus sequence of Sp1. Although Sp1 is known to be a general transcription factor, several studies indicate that it plays an important role in the up-regulation of genes under hypoxic conditions. For instance, Sp1 regulates expression of a disintegrin and metalloproteinase-17 (ADAM17) in U87 brain tumor cells in hypoxia.[31] A hypoxic environment increases nuclear localization of Sp1, with expression of the *cyclooxygenese-2* gene activated in human umbilical vein endothelial cells (HUVECs).[32] Hypoxia-induced up-regulation of deoxyribonuclease I (DNase I) expression in QGP-1 pancreatic cancer cells appears to be HIF-independent, and may be partially dependent on the Sp1 consensus sequence.[33] On the other hand, there is contradictory evidence that *IGF-2* transcription in hypoxic HepG2 cells depends on EGR-1, rather than Sp1.[29] Further studies are needed to clarify the relationships between the mechanism of action of furospinosulin-1 and the *IGF-2* transcription mechanism. In this regard, we pursued studies to identify the protein target of furospinosulin-1 binding.

Conclusions

In this study, we established a new screening system to discover compounds which can selectively inhibit proliferation of cancer cells under hypoxic conditions. The marine natural product furospinosulin-1 exhibited hypoxia-selective growth inhibition of cancer cells in vitro and exhibited in vivo antitumor activity without side effects upon oral administration. Mechanistic analysis of furospinosulin-1 revealed that its hypoxia-selective growth inhibition is a result of inhibition of *IGF-2* expression by prevention of complex formation between nuclear proteins and the Sp1 consensus sequence in the *IGF-2* promoter region (Figure 10). Moreover, IGF-2 was found to contribute significantly to the hypoxia-selective growth of cancer cells. We therefore propose that furospinosulin-1 is a new lead for anticancer drugs that target hypoxia-adapted cancer cells.

Experimental Section

Materials

RPMI 1640 and DMEM were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Monoclonal anti-VEGF, anti-IGF-2, anti-phosphotyrosine, anti-β-actin, and anti-PCNA antibodies were purchased from Abnova (Taipei, Taiwan), R&D systems (Minneapolis, MN, USA), Upstate (Billerica, MA, USA), Applied Biological Materials, Inc. (Richmond, BC, Canada), and Biolegend (San Diego, CA, USA), respectively. Polyclonal anti-IGF-1R and anti-IR antibodies were purchased from Signaling Antibody (Pearland, TX, USA) and Cell Signaling

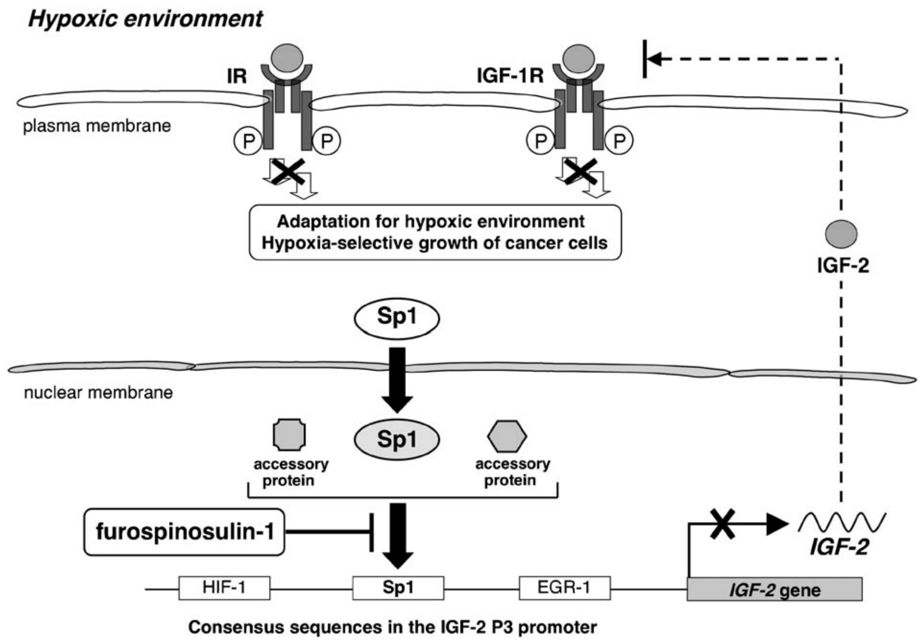

Figure 10. Proposed mechanism of action of furospinosulin-1.

Technology (Danvers, MA, USA), respectively. Monoclonal anti-HIF-1α antibody and protein G agarose beads were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Kanamycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Nacalai Tesque, Inc. (Kyoto, Japan).

Cell cultures

Human DU145 prostate cancer cells and S180 mouse sarcoma cells were cultured in RPMI 1640 supplemented with heat-inactivated 10% FBS and kanamycin (50 $\mu g\,mL^{-1}$) in a humidified atmosphere of 5% CO_2 at 37 °C. Human cervical cancer HeLa cells were grown in DMEM supplemented with 10% heat-inactivated FBS and kanamycin (50 $\mu g\,mL^{-1}$). The growth curves of these cells under hypoxic conditions were similar to those under normoxic conditions, respectively (figure S1, Supporting Information).

Isolation of furospinosulin-1

The methanol extract of the dried marine sponge (370 g) *Dactylospongia elegans* 01A10, which was collected in Indonesia in 2001, showed hypoxia-selective growth inhibition against DU145 cells. This methanol extract was partitioned into a H_2O–EtOAc mixture, with the EtOAc-soluble portion (54 g) further partitioned with a mixture of *n*-hexane and 90% MeOH. Based on bioassay guidance, the *n*-hexane-soluble portion (28 g) was fractionated by silica gel column chromatography (*n*-hexane/EtOAc) and ODS HPLC (Cosmosil 5C_{18}-MS-II, MeOH–H_2O) to afford furospinosulin-1 (65 mg) (Figure 1). Compound identification was confirmed by ESI-ToF-MS and 2D NMR spectroscopic analysis and compared with original spectral data.[15] Purity of the furospinosulin-1 used in biological studies was >99% (figure S2, Supporting Information).

Assay for antiproliferative activity under hypoxic conditions

Cells in the culture medium were plated in 96-well plates [1×10^4 cells per well (200 μL)] for 4 h in a humidified atmosphere of 5% CO_2 at 37 °C (normoxic conditions). The plates were incubated for 12 h in 94% N_2, 5% CO_2, and 1% O_2 (hypoxic conditions), inducing hypoxia-related genes such as HIF-1α. After incubation, compounds to be tested were added, and the plates were incubated for an additional 24 h under the same conditions. When anti-IGF-2 antibody was used as a test compound, DU145 cells were incubated for 24 h in RPMI 1640 supplemented with 1% FBS. Cell proliferation was detected according to an established MTT method, as previously described.[34] The growth inhibition rate was calculated as a percentage relative to negative controls. The antiproliferative activity of the test compounds was also evaluated under normoxic conditions using the MTT method.

In vivo antitumor effect of furospinosulin-1

All animal procedures were approved by the Committee on Animal Experimentation of Osaka University. S180 mouse sarcoma cells (1×10^6 cells per mouse) were implanted subcutaneously into the right ventral flank of female ddY mice (six weeks old). One week after implantation, furospinosulin-1 was administered orally every other day for two weeks (seven times total) as a suspension in 1% sodium carboxymethyl cellulose (CMC-Na). The remaining tumor was isolated and weighed to calculate the inhibition ratio. The control group and the group treated with furospinosulin-1 consisted of six mice each.

Western blotting analysis

A 40% confluent of DU145 cells in a six-well plate were pre-incubated for 12 h in a humidified atmosphere of 5% CO_2 at 37 °C (normoxic conditions) or 94% N_2, 5% CO_2, and 1% O_2 (hypoxic conditions). The cells were then treated with the indicated concentrations of furospinosulin-1 for 24 h under normoxic or hypoxic conditions. Each culture medium was transferred to a new test tube and incubated at 4 °C for 12 h in the presence of anti-VEGF antibody (1 $\mu g\,mL^{-1}$) or anti-IGF-2 antibody (1 $\mu g\,mL^{-1}$) to detect VEGF and IGF-2. Protein G agarose beads (100 μL) were added and the medium incubated for an additional 2 h at 4 °C. After removal of the medium by centrifugation, the beads were washed four times with lysis buffer [10 mM Tris·HCl (pH 7.5) containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 2 mM sodium vanadate, 1% SDS, 1% protease inhibitor cocktail-DMSO solution, and 1% phosphatase inhibitor cocktail-DMSO solution] and boiled in 2× sample buffer [100 mM Tris·HCl buffer (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue] for 5 min. A human IL-8 ELISA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to quantify IL-8 in the DU145 cell culture medium, according to the manufacturer's instructions.

The cells were rinsed with ice cold PBS and lysed in lysis buffer. To detect IGF-1R, phosphorylated IGF-1R, IR, and phosphorylated IR, equal amounts of cell lysates were incubated at 4 °C for 12 h in the presence of anti-IGF-1R antibody (1 $\mu g\,mL^{-1}$) or anti-IR antibody (1 $\mu g\,mL^{-1}$). Aliquot of the immunoprecipitates, trapped with protein G agarose beads, were boiled in 2× sample buffer for 5 min. The immunoprecipitates were resolved by 12% SDS-PAGE, transferred to PVDF membrane (GE Healthcare, Buckinghamshire, UK) and probed with anti-IGF-1R, anti-IR, and anti-phosphotyrosine antibodies to detect phosphorylated IGF-1R and IR. To detect HIF-1α and β-actin, the cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were probed with the appropriate primary antibodies. Immunopositive bands were visualized using an ECL kit (GE Healthcare, Buckinghamshire, UK).

Analysis of gene expression profile

DU145 cells (2×10^5 cells) were pre-incubated in a six-well plate for 4 h under normoxic conditions. The cells were then incubated for 12 h under normoxic or hypoxic conditions and either treated or not treated with 30 μM of furospinosulin-1 for an additional 12 h under normoxic or hypoxic conditions. The total RNA for each sample was isolated from the cells using an RNeasy Protect Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. RNA integrity was determined by UV/Vis spectroscopy and agarose gel electrophoresis.

A TrueLabeling-AMP 2.0 kit (SuperArray Bioscience Corp., Frederick, MD, USA) was used to reverse-transcribe mRNA to obtain cDNA. Each cDNA was then converted into biotinylated cRNA by in vitro transcription using biotin-16-UTP (Roche, Mannheim, Germany). Prior to hybridization, cRNA probes were purified with an ArrayGrade cRNA Cleanup kit (SuperArray Bioscience Corp.).

After purification, the 10 μg cRNA probes were hybridized with a microarray membrane (Oligo GEArray Hypoxia Signaling Pathway Microarray, SuperArray Bioscience Corp.). The expression levels of each gene were detected by chemiluminescence, using a copalyl diphosphate-star substrate (SuperArray Bioscience). Membranes were exposed to an X-ray film, and the signals were analyzed by using the GEArray Expression Analysis Suite (SuperArray Bioscience Corp.) with background subtracted. In this study, the cutoff values for determining altered gene expression as either up- or down-regulation were 1.5-fold and 0.65-fold, respectively.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from DU145 cells were prepared under normoxic or hypoxic conditions according to the method described by Milosevic et al.[35] Briefly, the DU145 cells were lysed in a hypotonic buffer [10 mM HEPES (pH 7.9) containing 10 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 2 mM sodium orthovanadate, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail], then the lysate was centrifuged at 2000 *g* for 5 min. The pelleted nuclei were resuspended in a hypertonic buffer [20 mM HEPES buffer (pH 7.9) containing 400 mM NaCl, 1.0 mM EDTA, 1.0 mM PMSF, 2 mM sodium orthovanadate, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail], followed by incubated on ice for 30 min. After the debris was removed by centrifugation, the resulting nuclear extracts were frozen at −80 °C until use. Protein concentration was determined by BCA assay (Pierce, Cleveland, OH, USA) and also confirmed by Western blotting with anti-PCNA antibody, which is a marker for nuclear proteins.

The 5′-biotinylated double-stranded oligonucleotides, which contained the binding sites of EGR-1 (5′-CCA CCC AGC CT <u>CGCCCCCGC</u> GCA CCC CCC A-3′), Sp1 (5′-CAG CCC GCA CC <u>CCCCGCCCCG</u> CTC TTG GCT-3′), and HIF-1 (5′-TGC GGG ATT TTG <u>TGCGT</u> GGT TTT TGA CTT G-3′), were synthesized by Hokkaido System Science Co. Ltd. (Hokkaido, Japan) and used as probes. The underlined nucleotides in each sequence are the consensus sequence of the corresponding transcription factor.

EMSA was performed using a LightShift chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions. Briefly, the nuclear proteins (2 μg) were pre-incubated on ice with binding buffer [10 mM Tris·HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1% (*v*/*v*) glycerol, 100 $\mu g\,mL^{-1}$ BSA, and 50 $\mu g\,mL^{-1}$ poly(deoxyinosine-deoxycytosine)] in the presence or absence of furospinosulun-1 (300 μM) for 20 min, then incubated with the labeled probe (4 fmol per 20 μL reaction mixture) for 15 min at room temperature. The reaction mixtures were separated on a 5% non-denaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 100 V for 60 min, then transferred to a nylon membrane (Hybond-N+, GE Healthcare). The membrane was incubated with LightShift-stabilized streptavidin–horseradish peroxidase conjugate, and the probe–protein complexes were visualized using an ECL kit.

Statistical analysis

Data are shown as the mean ±SD and represent the mean of at least three separate experiments performed in triplicate. Differences between data sets were determined by the Student's *t*-test. Those differences described as significant in the text correspond to $p<0.05$.

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[1] M. W. Dewhirst, Y. Cao, B. Moeller, *Nat. Rev. Cancer* **2008**, *8*, 425–437.
[2] B. G. Wouters, M. Koritzinsky, *Nat. Rev. Cancer* **2008**, *8*, 851–864.
[3] G. L. Semenza, *Oncogene* **2010**, *29*, 625–634.
[4] D. Kong, E. J. Park, A. G. Stephen, M. Calvani, J. H. Cardellina, A. Monks, R. J. Fisher, R. H. Shoemaker, G. Melillo, *Cancer Res.* **2005**, *65*, 9047–9055.
[5] A. L. Kung, S. D. Zabludoff, D. S. France, S. J. Freedman, E. A. Tanner, A. Vieira, S. Cornell-Kennon, J. Lee, B. Wang, J. Wang, K. Memmert, H. U. Naegeli, F. Petersen, M. J. Eck, K. W. Bair, A. W. Wood, D. M. Livingston, *Cancer Cell* **2004**, *6*, 33–43.
[6] T. W. Hodges, C. F. Hossain, Y. P. Kim, Y. D. Zhou, D. G. Nagle, *J. Nat. Prod.* **2004**, *67*, 767–771.

[7] K. A. Mohammed, C. F. Hossain, L. Zhang, R. K. Bruick, Y. D. Zhou, D. G. Nagle, *J. Nat. Prod.* **2004**, *67*, 2002 – 2007.
[8] K. A. Mohammed, R. C. Jadulco, T. S. Bugni, M. K. Harper, M. Sturdy, C. M. Ireland, *J. Med. Chem.* **2008**, *51*, 1402 – 1405.
[9] M. Y. Koh, T. Spivak-Kroizman, S. Venturini, S. Welsh, R. R. Williams, D. L. Kirkpatrick, G. Powis, *Mol. Cancer Ther.* **2008**, *7*, 90 – 100.
[10] L. A. Sonna, M. L. Cullivan, H. K. Sheldon, R. E. Pratt, C. M. Lilly, *Physiol. Genomics* **2003**, *5*, 195 – 207.
[11] A. Vengellur, J. M. Phillips, J. B. Hogenesch, J. J. LaPres, *Physiol. Genomics* **2005**, 22, 308 – 318.
[12] S. Aoki, Y. Watanabe, M. Sanagawa, A. Setiawan, N. Kotoku, M. Kobayashi, *J. Am. Chem. Soc.* **2006**, *128*, 3148 – 3149.
[13] S. Aoki, D. Kong, H. Suna, Y. Sowa, T. Sakai, A. Setiawan, M. Kobayashi, *Biochem. Biophys. Res. Commun.* **2006**, *342*, 101 – 106.
[14] X. Q. Ren, T. Furukawa, S. Aoki, T. Nakajima, T. Sumizawa, M. Haraguchi, Z. S. Chen, M. Kobayashi, S. Akiyama, *J. Biol. Chem.* **2001**, *276*, 23197 – 23206.
[15] G. Cimino, S. de Stefano, L. Minale, *Tetrahedron* **1972**, *28*, 1315 – 1324.
[16] D. Tasdemir, T. S. Bugni, G. C. Mangalindan, G. P. Concepcion, M. K. Harper, C. M. Ireland, *Z. Naturforsch. C* **2002**, *57*, 914 – 922.
[17] S. Urban, R. J. Capon, *Aust. J. Chem.* **1992**, *45*, 1255 – 1263.
[18] I. Erdogan-Orhan, B. Sener, S. de Rosa, J. Perez-Baz, O. Lozach, M. Leost, S. Rakhilin, L. Meijer, *Nat. Prod. Res.* **2004**, *18*, 1 – 9.
[19] D. LeRoith, C. T. Roberts, *Cancer Lett.* **2003**, *195*, 127 – 137.
[20] K. W. Kim, S. K. Bae, O. H. Lee, M. H. Bae, M. J. Lee, B. C. Park, *Cancer Res.* **1998**, *58*, 348 – 351.
[21] E. A. Bohula, M. P. Playford, V. M. Macaulay, *Anticancer Drugs* **2003**, *14*, 669 – 682.
[22] J. Rodon, V. DeSantos, R. J. Ferry, Jr., R. Kurzrock, *Mol. Cancer Ther.* **2008**, *7*, 2575 – 2588.
[23] T. Gloudemans, I. Prinsen, J. A. M. van Unnik, C. J. M. Lips, W. den Otter, J. S. Sussenbach, *Cancer Res.* **1990**, *50*, 6689 – 6695.
[24] D. Yee, K. J. Cullen, S. Paik, J. F. Perdue, B. Hampton, A. Schwartz, M. E. Lippman, N. Rosen, *Cancer Res.* **1988**, *48*, 6691 – 6696.
[25] G. K. Haselbacher, J. C. Irminger, J. Zapf, W. H. Ziegler, W. E. Humbel, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 1104 – 1106.
[26] E. Cariani, C. Lasserre, D. Seurin, B. Hamelin, F. Kemeny, D. Franco, M. P. Czech, A. Ullrich, C. Brechot, *Cancer Res.* **1988**, *48*, 6844 – 6849.
[27] M. A. van Dijk, F. M. A. van Schaik, H. J. Bootsma, P. Holthuizen, J. S. Sussenbach, *Mol. Cell. Endocrinol.* **1991**, *81*, 81 – 94.
[28] A. M. Raizis, M. R. Eccles, A. E. Reeve, *Biochem. J.* **1993**, *289*, 133 – 139.
[29] S. K. Bae, M. H. Bae, M. Y. Ahn, M. J. Son, Y. M. Lee, M. K. Bae, O. H. Lee, B. C. Park, K. W. Kim, *Cancer Res.* **1999**, *59*, 5989 – 5994.
[30] B. Dai, H. Wu, E. Holthuizen, P. Singh, *J. Biol. Chem.* **2001**, *276*, 6937 – 6944.
[31] A. Szalad, M. Katakowski, X. Zheng, F. Jiang, M. Chopp, *J. Exp. Clin. Cancer Res.* **2009**, *28*, 129 – 137.
[32] Q. Xu, Y. S. Ji, J. F. Schmedtje, Jr., *J. Biol. Chem.* **2000**, *275*, 24583 – 24589.
[33] Y. Kominato, R. Iida, T. Nakajima, Y. Tajima, R. Takagi, C. Makita, K. Kishi, M. Ueki, Y. Kawai, T. Yasuda, *Biochim. Biophys. Acta* **2007**, *1770*, 1567 – 1575.
[34] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55 – 63.
[35] J. Milosevic, P. Bulau, E. Mortz, O. Eickelberg, *Proteomics* **2009**, *9*, 1230 – 1240.

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