A novel mode of action of YC-1 in HIF inhibition: stimulation of FLH-dependent p300 dissociation from HIF-1α

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Abstract

Hypoxia-inducible factor (HIF)-1 plays a key role in tumor promotion by inducing ~60 genes required for tumor adaptation to hypoxia; thus, it is viewed as a target for cancer therapy. For this reason, YC-1, which down-regulates HIF-1α and HIF-2α at the post-translational level, is being developed as a novel anticancer drug. We here found that YC-1 acts in a novel manner to inhibit HIF-1. In the Gal4 reporter system, which is not degraded by YC-1, YC-1 was found to significantly inactivate the COOH-terminal transactivation domain (CAD) of HIF-1α, whereas it failed to inactivate CAD(N803A) mutant. In coimmunoprecipitation assays, YC-1 stimulated factor inhibiting HIF (FIH) binding to CAD even in hypoxia, whereas it failed to increase the cellular levels of hydroxylated Asn803 of CAD. It was also found that YC-1 prevented p300 recruitment by CAD in mammalian two-hybrid and coimmunoprecipitation assays. The involvement of FIH in YC-1-induced CAD inactivation was confirmed in EPO-enhancer and Gal4 reporter systems using FIH small interfering RNA and dimethyloxalylglycine FIH inhibitor. Indeed, FIH inhibition rescued HIF target gene expressions repressed by YC-1. In cancer cell lines other than Hep3B, YC-1 inhibits HIF-1α via the FIH-dependent CAD inactivation as well as via the protein down-regulation. Given these results, we suggest that the functional inactivation of HIF-1α contributes to the YC-1-induced deregulation of hypoxia-induced genes. [Mol Cancer Ther 2008;7(12):3729–38]

Introduction

Hypoxia is a common feature occurring in growing solid tumors and plays a pivotal role in tumor promotion, as hypoxia-driven genome changes promote tumor progression to more clinically aggressive phenotypes. This increased aggression is the result of the “turning-on” of the many genes required to overcome oxygen and nutrient deficiency and cell death. Hypoxic genome changes are mainly induced by hypoxia-inducible factors (HIFs), which function to control ≥60 hypoxia-induced genes (1). HIFs are heterodimeric transcription factors composed of HIF-α and HIF-β (also known as ARNT) subunits; HIF-1α is composed of HIF-1α/ARNT, and HIF-2α is composed of HIF-2α/ARNT. HIF-αs are prime transcription factors that transactivate genes, and ARNT assists HIF-α/DNA binding (2–4). Under normoxic conditions, HIF-αs are prolyl-hydroxylated by PHD enzymes, then ubiquitinated by von Hippel-Lindau protein, and consequently degraded by 26S proteasomes (5). HIF-αs are also regulated functionally by factor inhibiting HIF (FIH), a HIF asparagine hydroxylase. The transactivation domains of HIF-αs are hydroxylated by FIH and then cannot recruit p300/CBP coactivator, which leads to the repression of HIF-mediated transcriptional activity (6). In contrast, under hypoxic conditions, both proline and asparagine hydroxylation are inhibited; thus, HIF-αs are protected from von Hippel-Lindau protein binding and ubiquitination and activated by recruiting p300/CBP.

YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole] is a synthetic compound with a variety of pharmacologic actions (7). Because we showed the HIF-α-inhibitory activity of YC-1, YC-1 has been widely used as a pharmacologic tool for investigating the physiologic and pathologic roles of HIF (8). We also showed that YC-1 inhibits the growths of several human tumors xenografted in nude mice due to anti-HIF action (9). Subsequently, many researches have confirmed the anti-HIF and anticancer activities of YC-1 in vitro and in vivo (10). Moreover, the potential use of YC-1 has expanded to radiosensitization (11), tumor metastasis prevention (12), and choroidal neovascularization inhibition (13). Mechanistically, it has been found that YC-1 accelerates HIF-1α degradation by targeting its amino acids 720 to 780 region (14) or by inhibiting Mdm2 (15) and that it inhibits the de novo synthesis of HIF-1α by inactivating the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway (16). However, the precise mechanism that underlies HIF-α down-regulation by YC-1 remains uncertain.

In addition to HIF-α down-regulation, its functional inhibition might also offer a good cancer therapy strategy. For example, Kung et al. have found a functional inhibitor of HIF-α (chetomin) through a high-throughput screen.
This compound was identified to repress the transcriptional activity of HIF-α by disrupting p300 binding to HIF-α. Moreover, its anticancer activity was shown in xenografted human tumors (17). Bortezomib proteasome inhibitor is also known to have anticancer effect by inhibiting HIF-α. Bortezomib has been investigated clinically for the treatment of multiple myeloma and several solid tumors (18). In addition to its direct proapoptotic activity, bortezomib indirectly inhibits tumor angiogenesis by inactivating the COOH-terminal transactivation domain (CAD) of HIF-1α and subsequently repressing vascular endothelial growth factor (VEGF) expression (19, 20). Another proteasome inhibitor, MG132, also inactivates HIF-1α by stimulating the CITED2-mediated interference of CAD-p300 binding (21). Moreover, amphotericin B (an antifungal agent) inactivates HIF-1α by stimulating the CAD-FIH interaction, which may cause erythropoietin-deficient anemia associated with amphotericin B therapy (22). Given these reports, it appears that CAD inactivation could contribute to the down-regulation. In the present study, therefore, we questioned this mechanism having conducted many experiments using YC-1, because the efficacy of functional HIF inhibition by YC-1 is always superior to that of HIF-1 down-regulation by YC-1. In the present study, therefore, we addressed the possibility that the anti-HIF action of YC-1 is due in part to some mechanism other than HIF-α down-regulation.

Materials and Methods

Reagents and Antibodies
YC-1 was purchased from A.G. Scientific, and dimethylloxalylglycine (DMOG) was purchased from Frontier Scientific. Other chemicals were purchased from Sigma-Aldrich, and [α-32P]CTP (500 Ci/mmol) was purchased from GE Healthcare Bio-Sciences. Culture medium and FCS were purchased from Invitrogen. Anti-HIF-1α antiserum was raised in rabbits against glutathione S-transferase-tagged human HIF-1α (amino acids 418-698), and a monoclonal antibody against hydroxylated Asn803 of HIF-1α was raised in mouse immunized with a peptide containing a hydroxylated asparagine residue as described previously (9, 23). Other antibodies (anti-Gal4, anti-hemagglutinin (HA), anti-FIH, anti-p300, and anti-β-tubulin) were purchased from Santa Cruz Biotechnology.

Cell Culture
Hep3B (hepatoma), HEK293T (embryonic kidney), HT1080 (fibrosarcoma), and H1299 (non-small cell lung cancer) cell lines were obtained from the American Type Culture Collection and cultured in α-MEM or DMEM supplemented with 10% heat-inactivated FCS, respectively. Gas tensions in the O2/CO2 incubator (Vision SCI) were 20% O2/5% CO2 for normoxic incubation or 1% O2/5% CO2 for hypoxic incubation.

Expression Plasmids, Small Interfering RNAs, and Transfection
HA-tagged HIF-1α plasmid (pcDNA3), p300 plasmid, EPO-enhancer-luciferase plasmid, Gal4-CAD plasmid, and VP16-cysteine-histidine-rich domain 1 (CH1) plasmid were kindly provided by Dr. Eric Huang (University of Utah). The plasmid (sHIF-1α) used to stably express HIF-1α was made by deleting three degradation motifs (amino acids 397-405, 513-553, and 554-595) using a PCR-based mutagenesis kit (Stratagene). The Gal4-CAD plasmid was constructed by recombination of the HIF-1α CAD (amino acids 776-826) cDNA and the CMX-G4(N) plasmid, and the mutated Gal4-CAD(N805A) plasmid was generated by site-directed mutagenesis (24). The VP16-CH1 plasmid was constructed by recombination of the p300 CH1 cDNA and the VP16 plasmid (24). HA-tagged FIH plasmid, FIH small interfering RNA (siRNA), and green fluorescent protein were constructed as described previously (22). To express or to knock down genes, cells at 40% confluence in 60 mm dishes were transfected with plasmid or siRNA using calcium phosphate or Lipofectamine (Invitrogen) method.

Immunoblotting and Immunoprecipitation
For immunoblotting, cell lysates in a denaturing SDS sample buffer were electrophoresed on 8% to 12% SDS-polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore). Nonspecific proteins on membranes were blocked in 5% skim milk TBS-Tween 20 for 1 h and then incubated overnight with the primary antibodies, diluted 1:200 to 1:1,000 in the blocking solution. After washing, the membranes were incubated with the secondary antibodies conjugated with horseradish peroxidase (1:5000) for 1 h, and the immune complexes were visualized using the ECL-Plus kit (GE Healthcare Bio-Sciences). For immunoprecipitation, cells were cotransfected with 4 μg each of plasmids (HA-FIH, sHIF-1α, or p300). Forty-two hours after transfection, the cells were solubilized, and the cell lysates were incubated with an antibody for precipitation for 4 h and then incubated with protein A/G-Sepharose beads (GE Healthcare Bio-Sciences) overnight at 4°C. After washing, the precipitated proteins were eluted in the denaturing SDS sample buffer and then subjected to SDS-PAGE and immunoblotting using an antibody for identification.

Reporter Assays
Cells were cotransfected with 1 μg each of reporter gene and plasmid cytomegalovirus-β-galactosidase (β-gal) using the calcium phosphate method. pcDNA was added to ensure that the final DNA concentrations in both control and experimental groups were at similar levels. After stabilized for 24 h, cells were incubated under either normoxic or hypoxic conditions, in the absence or in the presence of YC-1 for 16 h, and then lysed to determine luciferase and β-gal activities.

Semiquantitative Reverse Transcription-PCR for EPO or VEGF mRNA
To quantify mRNA levels, we used a highly sensitive semiquantitative reverse transcription-PCR as described...
previously (22). Total RNAs, extracted using Trizol (Invitrogen), were reverse transcribed at 46°C for 20 min and the cDNAs were amplified over 18 (for VEGF) or 28 (for EPO) PCR cycles (94°C for 30 s, 53°C for 30 s, and 70°C for 30 s) in a 20 μL reaction mixture containing 5 μCi [α-32P]dCTP. The PCR products were electrophoresed on a 4% polyacrylamide gel in 1× Tris-acetate-EDTA solution, and dried gels were autoradiographed. Primers for human EPO, VEGF, and β-actin were designed as described previously (22).

Statistics

All data were analyzed using Microsoft Excel 2002 software, and results are expressed as means and SDs. We used the unpaired Student’s t test to compare reporter activities between control and YC-1-treated groups. Differences were considered significant when

Results

YC-1 Inhibited HIF Activity More Than It Reduced HIF-α Protein Levels

As reported previously (8), YC-1 reduced the protein levels of HIF-1α and HIF-2α under hypoxic conditions in a dose-dependent manner (Fig. 1A). Also, YC-1 repressed EPO-enhancer activity, which is determined by the transcriptional activities of HIF-1α and HIF-2α, also in a dose-dependent manner (Fig. 1B). Although protein suppression and activity repression showed similar patterns, we formed the impression (after performing several experiments) that functional HIF inhibition by YC-1 is greater than that indicated by HIF suppression at the protein level. Therefore, we compared degrees of functional inhibition with HIF-1α and HIF-2α protein suppressions. It was found that the functional activities of HIF tend to be ~20% less HIF-1α and HIF-2α protein levels for all YC-1 doses examined (Fig. 1C). Thus, we suggest that some another mechanism, in addition to HIF-1α and HIF-2α down-regulation, underlies the anti-HIF effect of YC-1. Therefore, we hypothesized that YC-1 functionally inactivates HIF.

YC-1 Inactivated the Transactivation Domain of HIF-1α Dependently of Asn803

To exclude the influence of HIF-α down-regulation by YC-1, we used the Gal4-CAD/Gal4-luciferase reporter, the activity of which is determined only by the transactivation domain of HIF-1α and not by endogenous HIF. Moreover, because Gal4-CAD protein does not contain the degradation domain of HIF-α, we expected it to be stably expressed. In the event, as was expected, Gal4-CAD protein levels were unaffected by either hypoxia or YC-1 treatment (Fig. 2A). However, the transcriptional activity of

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Gal4-CAD was definitely repressed by YC-1 in a dose-dependent manner (Fig. 2B), which suggests that YC-1 functionally inhibits the transactivation domain of HIF-1α. As mentioned in Introduction, the FIH-mediated inactivation of CAD is the key step in the oxygen-dependent repression of HIF-1. To test the possibility that the YC-1-mediated repression of CAD is linked with such a regulation mechanism, we examined the effect of YC-1 on the activity of mutated CAD lacking Asn803, the target of FIH. We first confirmed that Gal4-CAD(N803A) protein expression was not affected by YC-1 (Fig. 2C) and then measured Gal4-CAD(N803A) activities. Interestingly, YC-1 failed to inactivate the mutated CAD as shown in Fig. 2D. Taken together, these results suggest that YC-1 inactivates the transactivation domain of HIF-1α in an Asn803-dependent manner.

**YC-1 Stimulated FIH Binding to HIF-1α CAD**

Because FIH binding to CAD is the key step in Asn803-dependent CAD regulation, we examined whether YC-1 stimulates FIH binding. Accordingly, we coexpressed Gal4-CAD and HA-tagged FIH in HEK293 cells and performed communoprecipitation (Fig. 3A). Amounts of Gal4-CAD and FIH were not affected by YC-1, but FIH-CAD binding was lower in hypoxic cells than in normoxic cells, which suggests that FIH regulates HIF-1α oxygen-dependently. However, YC-1 noticeably recovered FIH binding in hypoxic cells, suggesting that YC-1 reinforces FIH binding to HIF-1α CAD even under hypoxic conditions. Because FIH is known to hydroxylate Asn803, we examined whether YC-1 stimulates the Asn803 hydroxylation using a monoclonal antibody that specifically detects hydroxylated Asn803 (Fig. 3B). As expectedly, the hydroxylation was noticeably reduced in hypoxia and stimulated by FIH overexpression, which verifies the specificity of this antibody. However, YC-1 failed to increase the cellular levels of hydroxylated Asn803. Despite increasing FIH binding to CAD, YC-1 is unlikely to stimulate the catalytic activity of FIH.

**YC-1 Inhibited p300 Recruitment by HIF-1α CAD**

FIH is known to inactivate CAD by preventing the interaction between CAD and the CH1 domain of p300 coactivator. Thus, we examined the CAD-p300 interaction using a mammalian two-hybrid assay, in which reporter activity depends on the interaction between Gal4-CAD and VP16-CH1. Figure 4A shows that the CAD-CH1 interaction was enhanced by hypoxia and that this interaction was significantly inhibited by YC-1. We also analyzed the interaction between Gal4-CAD mutant (N803A) and VP16-CH1 and found that YC-1 failed to inhibit CAD(N803A)-CH1 binding (Fig. 4B). To confirm HIF-1α-p300 dissociation...
**Figure 3.** Effects of YC-1 on FIH-CAD binding and Asn803 hydroxylation. A, FIH-CAD binding. HEK293 cells were cotransfected with HA-FIH (2 μg) and Gal4-CAD (2 μg) plasmids and incubated under normoxic or hypoxic (16 h) conditions in the absence or presence of YC-1 (5 μmol/L). Protein amounts in the samples were verified by Western blotting (top). Immunoprecipitations were done using anti-Gal4 antiserum (αGal4) or nonimmunized serum (IgG), and the coprecipitation of HA-FIH with Gal4-CAD was identified by Western blotting using anti-FIH antiserum (middle). The FIH-CAD binding was cross-checked by changing antisera (bottom). B, Asn803 hydroxylation. Hep3B cells were transfected with HA-FIH (2 μg) and/or shHIF-1α (2 μg) plasmids and incubated under normoxic or hypoxic (16 h) conditions in the absence or presence of YC-1 (20 μmol/L). Protein amounts were analyzed by Western blotting. Asn803 hydroxylated HIF-1α was detected using an antibody against hydroxylated asparagine, αN803(OH).

By YC-1, we expressed p300 and stable HIF-1α lacking two prolyl hydroxylation motifs. p300 was immunoprecipitated using anti-p300 antibody, and HIF-1α coprecipitation was identified using anti-HIF-1α antiserum (Fig. 4C). HIF-1α-p300 interaction was enhanced by hypoxia, and this interaction was noticeably inhibited by YC-1. These results suggest that YC-1 inhibits the recruitment of p300 by HIF-1α under hypoxic conditions via a FIH-dependent mechanism.

**FIH Inhibitions Recover HIF Activity Inhibited by YC-1**

To confirm the involvement of FIH in HIF inactivation by YC-1, we examined whether CAD activity is rescued by FIH inhibition. For this purpose, we treated cells with FIH siRNA or DMOG (a functional inhibitor of FIH). The FIH-silencing effect of the siRNA was verified by checking the cellular levels of FIH protein (Supplementary Fig. S1). Figure 5A shows that both FIH siRNA and DMOG significantly prevented the YC-1 inactivation of CAD, which supports the FIH dependency of the action of YC-1. Next, to examine how much FIH-mediated CAD inactivation contributes to the HIF-inhibitory effect of YC-1, we analyzed the transcriptional activities of endogenous HIFs in siRNA- or DMOG-treated cells. Both FIH inhibitions were found to partially but significantly rescue the CAD activity inhibited by YC-1 (Fig. 5B). Because YC-1 either down-regulates or inactivates HIF-α, HIF-α reactivation by FIH inhibition appeared to partially recover CAD activity. In Fig. 5C, we summarize results regarding FIH activity and HIF-α down-regulation. In addition, a 25% to 30% difference in the HIF-inhibitory effect of YC-1 was observed for HIF-α protein levels and HIF activity in control siRNA-treated cells. However, the CAD-inhibiting effect of YC-1 was eliminated in FIH knocked down cells; thus, such differences might be abolished (Fig. 5C). To examine whether the FIH activation by YC-1 is involved in real gene suppression by YC-1, we analyzed EPO and VEGF mRNA levels. Both were noticeably induced under hypoxic conditions, and these inductions were almost completely abolished by YC-1 treatment. As was expected, both FIH siRNA and DMOG effectively prevented YC-1-repressing EPO and VEGF but did not fully recover these gene expressions (Fig. 5D). This result well matched the effects of FIH inhibition on reporter activities, as shown in Fig. 5B, and further supports the notion that FIH-mediated HIF-α inactivation contributes to the YC-1 suppression of HIF target genes.

**YC-1 Inhibits HIF-1 Function in Cancer Cell Lines Other Than Hep3B**

To examine whether anti-HIF action of YC-1 is a general event, we treated HT1080 and H1299 cells with various concentrations of YC-1 and found that YC-1 also down-regulated HIF-1α (Fig. 6A). However, a higher concentration (100 μmol/L) of YC-1 was required for significant suppression of HIF-1α in these cells. In the EPO-enhancer reporter assay, YC-1 started to inhibit the transcriptional activity of HIF-1 at 5 μmol/L and further inhibited it at higher concentrations (Fig. 6B). We also compared the reporter activities with HIF-1α levels and found that HIF-1 activities tend to be 10% to 40% less than HIF-1α levels (Fig. 6C). Furthermore, YC-1 noticeably inhibited the hypoxic induction of VEGF and the FIH knockdown partially recovered VEGF expression inhibited by YC-1 in both cell lines (Fig. 6D). These results suggest that, in cancer cells other than Hep3B, YC-1 inhibits HIF-1α via the FIH-dependent CAD inactivation as well as via the protein down-regulation. The effect of YC-1 on HIF-1α CAD seems to occur generally in cancer cells but is somewhat cell type specific in an aspect of drug sensitivity.
Discussion

In the present study, we tested the possibility that, in addition to HIF-1α down-regulation, the functional inactivation of HIF-1α also contributes to the YC-1-induced deregulation of hypoxia-induced genes. In brief, YC-1 stimulated FIH binding to HIF-1α CAD, which in turn prevented p300 binding to CAD and led to the functional repression of HIF. The mechanisms underlying HIF inhibition by YC-1 are summarized in Supplementary Fig. S2.5

FIH was first identified as a HIF-1α CAD-interacting protein by yeast two-hybrid screening (25), and its function has been extensively investigated. FIH is an enzyme that hydroxylates the Asn803 residue in HIF-1α and which belongs to the 2-oxoglutarate-dependent dioxygenase superfamily (26, 27). Like other dioxygenases, FIH requires O2, Fe2+, and 2-oxoglutarate for its enzymatic action. Furthermore, the DMOG used in this study is known to inhibit FIH by competing with 2-oxoglutarate (27, 28). The action of FIH and its X-ray crystal structure are well understood, but little is known about the systems that regulate the FIH-HIF-1α interaction or FIH enzymatic activity. In the present study, we found that YC-1 stimulates the FIH-HIF-1α interaction and then inactivates HIF-1α under hypoxic conditions. Moreover, since YC-1 did not affect the cellular levels of FIH (Fig. 5A), we speculate that YC-1 either activates a positive regulator or inhibits a negative regulator in the FIH-HIF-1α interaction. However, we do not know how YC-1 stimulates the FIH binding, and the precise mechanism involved remains to be addressed by future studies.

p300 coactivator binds with HIF-1α proteins through its CH1 region (24). p300 functions as a scaffold that enables HIF-1α to form a transcription complex with TBP, TFIIIB, and RNA polymerase II. In addition, the HAT domain of p300 facilitates transcription complex access to DNA by acetylating promoter-proximal nucleosomal histones (29). Therefore, p300 CH1 binding to HIF-1α CAD is an initial event that is essential for gene expression by HIF-1, and HIF-1α activity is critically controlled by p300 binding. Physiologically, HIF-1α-p300 binding is known to be regulated in two distinct ways: by hydroxylation of Asn803 in CAD and by direct competition to p300 binding. The former is activated oxygen-dependently by FIH (6) and the latter is processed oxygen-independently by CITED2 (alternatively named Mrg1 or p35srj), which stimulates the FIH-HIF-1α interaction and then inactivates HIF-1α under hypoxic conditions.
has an affinity for p300 that is much higher than that of HIF-1α (30). Then, which one of these two mechanisms is responsible for p300 dissociation by YC-1? To answer this question, we analyzed the cellular levels of hydroxylated Asn803 and identified that the Asn 803 hydroxylation was not stimulated by YC-1. From this result, we can rule out the former possibility and thus consider the latter as the mechanism underlying CAD inactivation. That is, YC-1 inactivates HIF-1α CAD by competition to p300 binding.

Then, which molecule competes with p300 in CAD binding? Our mammalian two-hybrid assay using Gal4-CAD(N803A) plasmid might exclude the involvement of CITED2 in the YC-1 effect. Gal4-CAD(N803A) stably binds to p300 and thus constitutively expresses luciferase. However, Gal4-CAD(N803A) activity is still regulated by CITED2, because CITED2 deprives HIF-1α of p300 binding. In this respect, p300 dissociation by YC-1 is not likely to be mediated by CITED2, because Gal4-CAD(N803A) was active even in the presence of YC-1. Alternatively, FIH could be another potential competitor of p300 in HIF-1α binding. As it is a short segment composed of 50 amino acids, CAD may not have a space enough to bind both FIH and p300 at the same time; thus, FIH could compete with p300 in CAD binding regardless of its hydroxylation of Asn803. If so, FIH-CAD binding per se may be responsible for the YC-1-induced disruption of p300-CAD binding. However, it should be noted that this explanation is just speculated but not based on evidence. The molecular mechanism by which YC-1 disrupts p300-CAD interaction remains to be investigated.

Besides HIF-α, some other proteins harboring containing ankyrin repeat domain (ARD) have been reported to be targeted by FIH as well. Cockman et al. have identified a series of ARD-containing proteins as FIH-interacting proteins through yeast two-hybrid screening (31). Of screened ARD proteins, nuclear factor-κB p105 and IκBα were shown to be hydroxylated at Asn residues by p300.
expressed FIH. Recently, another ARD proteins, Notch receptors, were also reported to be hydroxylated by FIH (32, 33). However, because FIH did not regulate nuclear factor-κB or Notch transcriptional response, the significance of FIH-mediated hydroxylation remains unclear in ARD proteins other than HIF-1α. Given that YC-1 reinforced the FIH inhibition to HIF-1α transcriptional response, perhaps YC-1 could affect the activities of these ARD proteins targeted by FIH. Thus, we analyzed the transcriptional activity of nuclear factor-κB and the repressive activity of IκBα using a luciferase reporter and the IκBα plasmid. However, YC-1 failed to affect the transcriptional responses by nuclear factor-κB and IκBα (Supplementary Fig. S3), which suggests that the FIH-dependent regulation by YC-1 does not occur universally in ARD proteins, rather uniquely in HIF-1α.

YC-1 was originally characterized as a cGMP inducer because it stimulated soluble guanylyl cyclase activation in response to nitric oxide or carbon monoxide (34, 35). As a result of cGMP elevation, the pharmacologic actions of YC-1 include anticoagulation, vascular dilatation, and penile erection (7). In addition to these effects, it has also been found that YC-1 has anti-HIF (8) and antiproliferative (36) activities in cancer cells. In some cancer cells, only low concentrations of YC-1 (1-20 μmol/L) are required for anti-HIF activity, whereas cGMP elevation requires higher concentrations (>50 μmol/L; ref. 36). Therefore, if the dose of YC-1 is well adjusted, it is believed that YC-1 can be used to specifically inhibit HIF without the cGMP-dependent side effects in YC-1-sensitive tumors such as hepatoma. However, compared with Hep3B (EC50 = ~2 μmol/L), HT1080 and H1299 showed a lower sensitivity in HIF inhibition by YC-1 (EC50 = ~50 μmol/L). Because 50 μmol/L YC-1 is so high as to stimulate cGMP production, the cGMP-dependent side effects, including increased bleeding time and hypotension, can be expected.

In this respect, the anticancer usage of YC-1 may be limited to YC-1-sensitive tumors and this should be considered during the preclinical trial of YC-1. Then, why do the YC-1 sensitivity different? In general, different pharmacokinetic properties (penetration, excretion, or metabolism) and characters of target molecules (expression,
activation, or isoforms) are considered as major factors determining drug sensitivity. However, these factors regarding YC-1 have been underdetermined to date; thus, the cell type-dependent sensitivity to YC-1 remains to be investigated.

Many clinical and animal studies have disclosed that HIF-1α plays positive roles in tumor angiogenesis, growth, and metastasis (1, 37). Inversely, HIF-1α has been suggested to induce growth arrest and apoptosis by up-regulating cyclin-dependent kinase inhibitors and proapoptotic factors (38, 39). HIF-1α may function as a double-edged sword in tumor response to hypoxia, and how the cell fate is determined by HIF-1α remains to be answered to date. Regarding this question, it can be noted that the cellular adaptation to hypoxia mainly depends on the transcriptional activity of HIF-1α. In contrast, the tumor-inhibiting effect of HIF-1α can be brought about independently of its transcriptional activation; that is, it is attributable to HIF-1α binding to p53, MAX, and β-catenin (40–42). Moreover, the NH2-terminal half and ODD domain of HIF-1α parts participate in these protein bindings, but CAD does not. If so, the CAD inactivation could be expected to make a deeper effect on hypoxia adaptation than on growth inhibition, leading to impaired angiogenesis and growth inhibition in hypoxic tumors. In this respect, the YC-1 inactivation of CAD could be a potential strategy for treating tumors with high levels of HIF-1α.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

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