YC-1 Induces S Cell Cycle Arrest and Apoptosis by Activating Checkpoint Kinases

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Abstract

Hypoxia-inducible factor-1α (HIF-1α) seems central to tumor growth and progression because it up-regulates genes essential for angiogenesis and the hypoxic adaptation of cancer cells, which is why HIF-1α inhibition is viewed as a cancer therapy strategy. Paradoxically, HIF-1α also leads to cell cycle arrest or the apoptosis of cancer cells. Thus, the possibility cannot be ruled out that HIF-1α inhibitors unlock cell cycle arrest under hypoxic conditions and prevent cell death, which would limit the anticancer effect of HIF-1α inhibitors. Previously, we reported on the development of YC-1 as an anticancer agent that inhibits HIF-1α. In the present study, we evaluated the effects of YC-1 on hypoxia-induced cell cycle arrest and cell death. It was found that YC-1 does not reverse the antiproliferative effect of hypoxia, but rather that it induces S-phase arrest and apoptosis at therapeutic concentrations that inhibit HIF-1α and tumor growth; however, YC-1 did not stimulate cyclic guanosine 3′,5′-monophosphate production in this concentration range. It was also found that YC-1 activates the checkpoint kinase–mediated intra-S-phase checkpoint, independently of ataxia-telangiectasia mutated kinase or ataxia-telangiectasia mutated and Rad3-related kinase. These results imply that YC-1 does not promote the regrowth of hypoxic tumors because of its cell cycle arrest effect. Furthermore, YC-1 may induce the combined anticancer effects of HIF-1α inhibition and cell growth inhibition. (Cancer Res 2006; 66(12): 6345-52)

Introduction

YC-1 [3-(5-hydroxymethyl-2′-furyl)-1-benzylindazole] activates soluble guanylate cyclase and was originally developed as a drug to inhibit platelet aggregation or to prevent vascular contraction (reviewed in ref. 1). Currently, it is also used as an inhibitor of hypoxia-inducible factor 1α (HIF-1α). YC-1 was found to reduce the protein level of HIF-1α and to inhibit the expression of hypoxia-inducible genes in cultured hepatoma cells (2). In vivo, YC-1 halted the growth of five xenografted human tumors without inducing apparent toxicities. Moreover, tumors from YC-1-treated mice showed fewer blood vessels, reduced HIF-1α levels, and lower levels of HIF-1-regulated gene transcription (3). Thus, YC-1 is regarded as a good lead compound for the development of HIF-1-targeting anticancer agents (4).

HIF-1α, a basic-helix-loop-helix Per-Arnt-Sim transcription factor, functions as a master regulator of oxygen homeostasis. Under normoxic conditions, HIF-1α is modified by HIF-1-prolyl hydroxylyases and then degraded by pVHL-mediated ubiquitination. Under hypoxic conditions, HIF-1α becomes stabilized and this stabilization leads to tumor survival via increased angiogenesis and anaerobic glycolysis (reviewed in ref. 5). Paradoxically, HIF-1α also up-regulates genes that promote cell cycle arrest and apoptosis, such as p21, p27, p53, Nip3, Noxa, and HGTDP (6, 7). Thus, HIF-1α seems to be a double-edged sword in tumor cells subjected to hypoxia and may determine the fates of hypoxic cells. In this respect, the benefits of HIF-1α inhibition in cancer therapy remain questionable as it can unlock cell cycle arrest under hypoxic conditions and prevent cell death. These contrary effects may limit the anticancer effect of HIF-1α inhibitors, as ostensibly the ideal agent should block only hypoxic adaptation. Furthermore, if HIF-1α inhibitors have the additional effect of inducing cell cycle arrest or cell death, they might be better propositions as anticancer agents. Thus, it seems that in terms of developing HIF-1α inhibitors as anticancer agents, it is necessary to evaluate their effects on the cell cycle and cell death.

Although we showed the anticancer effect of YC-1 in a previous study (3), we did not evaluate the potential adverse effects of YC-1 on hypoxia-induced cell cycle arrest and cell death. Thus, here, we examined these effects of YC-1. Surprisingly, YC-1 showed the additional effects of cell cycle arrest and tumor growth inhibition at therapeutic concentrations that inhibit HIF-1α. Moreover, YC-1 induced S-phase arrest, which was followed by apoptosis, and activated the checkpoint kinase 1/2 (Chk1/2)–mediated intra-S-phase checkpoint. We conclude that this cell cycle arrest in combination with HIF-1α inhibition is likely to contribute to the anticancer effect of YC-1.

Materials and Methods

Reagents. YC-1 was purchased from A.G. Scientific, Inc. (San Diego, CA) and dissolved in DMSO to produce a 100 mmol/L stock solution. Hydroxyurea, 3′-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, propidium iodide, RNase A, and the chemical components of buffers were purchased from Sigma-Aldrich (St. Louis, MO). All culture medium and fetal bovine serum were purchased from Life Technologies (Grand Island, NY) and antibiotics were from Invitrogen (Carlsbad, CA).

Cell culture. Hep3B hepatoma, HEK293 embryonic kidney, and Caki-1 renal carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Hep3B cells were cultured in α-modified Eagle medium, whereas HEK293 and Caki-1 cells were cultured in DMEM. All culture media were supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL), and all cells were grown in a humidified atmosphere containing 5% CO2 at 37°C. Oxygen tensions in the...
incubator were either 140 mm Hg (20% O₂ v/v, normoxia) or 3.5 mm Hg (0.5% O₂ v/v, hypoxia).

**Cell proliferation and viability assays.** Bromodoxouridine (BrdUrd) incorporation assays were done using a FITC BrdUrd flow kit provided by BD Pharmingen (San Diego, CA). Briefly, after normoxic or hypoxic incubation, cells were treated with 10 μmol/L BrdUrd for 30 minutes. After fixation and permeabilization, cells were treated with DNase and incubated with FITC-conjugated anti-BrdUrd (BD Pharmingen). Total DNAs were stained with 7-aminomethylcoumarin D. FITC and 7-amino-actinomycin D were excited with an argon laser at 488 nm and detected at 515 to 565 nm and 630 to 660 nm, respectively, using a FACStar flow cytometer (BD Biosciences, San Jose, CA). Cell viabilities were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, after incubation with DMSO or YC-1, MTT was added to the medium (0.5 mg/mL) and incubated at 37°C for 3 hours. The resulting insoluble formazan was dissolved with 0.04 N HCl in isopropanol and measured at 570 nm using a spectrophotometer.

**Cell cycle analysis.** Cells were incubated until a concentration of 70% to 80% confluence. After treatment with DMSO or YC-1, cells were harvested and fixed in 75% ethanol for 30 minutes on ice. After washing with PBS, cells were labeled with propidium iodide (0.05 mg/mL) in the presence of RNase A (0.5 mg/mL) and incubated at room temperature in the dark for 30 minutes. DNA contents were analyzed using a flow cytometer. Propidium iodide incorporated into DNA was excited at 488 nm and detected at 650 nm.

**Preparation of small interfering RNA and transfection.** To knock down HIF-1α, Chk1, Chk2, ataxia-telangiectasia mutated (ATM) kinase, or ATM- and Rad3-related (ATR) kinase, synthesized small interfering RNA (siRNA) duplexes were obtained from Invitrogen. The siRNA sequences corresponded to nucleotides (the coding region) 360 to 384 of HIF-1α (Genbank accession no. NM_001530), 477 to 494 of Chk1 (NM_001274), 250 to 268 of Chk2 (NM_007194), 427 to 447 of ATM (NM_000051), or 189 to 209 of ATR (NM_001184). For siRNA transfection, 40% of the confluent cells in 60 mm cell culture dishes were respectively transfected with these siRNAs using the calcium phosphate methodology. The transfected cells were then allowed to stabilize for 48 hours before being used in experiments.

**Measurements of apoptosis.** Apoptotic cell death was analyzed using three different methods: by determining caspase-3 activity or poly(ADP-ribose) polymerase (PARP) cleavage, or by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL). Caspase-3 activity was measured using a caspase-3 assay kit provided by Sigma-Aldrich. Briefly, detached cells were centrifuged and resuspended in 100 μL lysis buffer [50 mmol/L HEPES (pH 7.4), 5 mmol/L CHAPS, and 5 mmol/L DTT]. Lysates were incubated with 0.2 mmol/L Ac-DEVD-pNA at 37°C for 20 hours, and caspase-3 activities were measured at 410 nm in the presence or absence of the caspase-3 inhibitor Ac-DEVD-CHO (1 mmol/L). Specific activities are presented in nanomoles of paranitroaniline released from 1 mg protein/min. For PARP cleavage detection, cells were lysed in a buffer containing 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 20 mmol/L EDTA, 100 μmol/L NaF, 10 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L leupeptin, 20 μg/mL aprotinin, and 1% Triton X-100, and 25 μg of the protein thus obtained was separated by 8% SDS-PAGE and subsequently transferred to an Immobilon-P membrane (Millipore, Bedford, MA). After blocking nonspecific binding sites with 5% nonfat milk, membranes were incubated with anti-PARP antibody (Biomol Research Laboratories, Plymouth Meeting, PA) at a dilution of 1:5,000 (in 5% nonfat milk). Horseradish peroxidase (HRP)–conjugated anti-mouse antibody (Zymed Laboratories, South San Francisco, CA) was used as a secondary antibody (1:5,000 dilution in 5% nonfat milk). Antigen-antibody complexes were visualized using an Enhanced Chemiluminescence Plus kit (Amersham Biosciences, Piscataway, NJ). TUNEL assays were done using the In situ Cell Death Detection kit provided by Roche Applied Science (Mannheim, Germany). Cells were then harvested, fixed with 2% parformaldehyde for 1 hour, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 5 minutes on ice. To measure fragmented DNA levels, cells were incubated in the TUNEL reaction mixture with terminal deoxynucleotidyl transferase and FITC-dUTP at 37°C for 1 hour and stained with 0.05 mg/mL propidium iodide. Fluorescein incorporated into DNA was excited at 488 nm and detected at 550 nm.

**Immuno blot analyses.** Total cell lysates (40 μg protein) were separated on 4% to 12% SDS-polyacrylamide gels, and blots were transferred to Immobilon-P membranes (Millipore). Membranes were blocked with 5% nonfat milk in TBS containing 0.1% Tween 20 (TTBS) at room temperature for 1 hour and then incubated at 4°C overnight with a primary antibody (diluted 1:1,000), i.e., anti-cyclin D1, anti-Chk1, anti-phospho-Chk1(Ser317), anti-Chk2, or anti-phospho-Chk2 (Thr382). Cell Signaling Technology, Beverly, MA), or anti-ATM (GeneTex, Inc., San Antonio, TX), or anti-phospho-ATM (Ser1981; Rockland Immunocchemicals, Gilbertsville, PA), or anti-HIF-1α (3) in 5% nonfat milk in TTBS. HRP-conjugated anti-mouse or anti-rabbit antisera was used as a secondary antibody (1:5,000 dilution, 2-hour incubation) and antigen-antibody complexes were visualized using an Enhanced Chemiluminescence Plus kit. The levels of tubulin and β-actin were measured using rabbit polyclonal anti-β-tubulin antibody and mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as loading controls.

**Comet assay.** DNA breakage was analyzed by Comet assay. Hep3B cells were treated with YC-1 or H₂O₂ (to provide a positive control), and then embedded in agarose on microscope slides, lysed, and electrophoresed. DNA fragments were quantitated by single- or double-stranded breaks migrated faster than intact DNA. DNA samples were stained with SYBR green dye. The DNA fragments were visible as Comet tails by fluorescence microscopy.

**Nuclear protein extraction and topoisomerase activity assay.** Hep3B cells were treated with YC-1 and quickly washed in ice-cold PBS. Scraped cells were centrifuged at 800 × g for 5 minutes at 4°C, and the cell pellets were resuspended in 10 packed cell volumes of a lysis buffer [20 mmol/L Tris (pH 7.8), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.2 mmol/L EDTA containing 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and 1 mmol/L Na3VO4]. The plasma membranes were disrupted by adding NP40 (final 0.6%). After centrifugation, the nuclear pellets were resuspended in three packed pellet volumes of a hypertonie solution containing 5% glycerol and 400 mmol/L NaCl in the lysis buffer. After incubating on ice for 30 minutes, cells were centrifuged and the supernatants (containing nuclear proteins) were kept at −70°C.

**Topoisomerase I activities were determined using DNA relaxation assays.** Nuclear proteins (0.2 μg) were incubated at 37°C for 30 minutes with 0.2 μg of pcDNA in 20 μL of a reaction buffer [10 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L DTT, 1 mmol/L EDTA, and 0.1 mg/mL bovine serum albumin (BSA)]. The reaction was stopped by adding 4 μL of a stop buffer/loading dye mixture (5% Sarkosyl, 0.025% bromphenol blue, and 25% glycerol). Products were electrophoresed on 0.8% agarose gels and stained with ethidium bromide. Topoisomerase II activities were analyzed using a Eugaryotic Topo II assay kit (TopoGEN, Inc., Port Orange, FL), which detected the ATP-dependent decatenation of kinetoplast DNA. Nuclear proteins (1 μg) were incubated at 37°C for 30 minutes with 145 ng kinetoplast DNA in 20 μL reaction buffer [50 mmol/L Tris (pH 8.0), 120 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L ATP, 0.5 mmol/L DTT, and 30 μg/mL BSA]. The reaction was stopped after adding 4 μL stop buffer/loading dye mixture. DNA samples were electrophoresed on 1% agarose gels and then stained with ethidium bromide.

**Cyclic guanosine 3′,5′-monophosphate assay.** Cyclic guanosine 3′,5′-monophosphate (cGMP) levels were measured using an immunoassay kit provided by Amersham Pharmacia Biotech (Piscataway, NJ). Briefly, Hep3B cells were lysed with 5% dodecyltrimethylammonium bromide and incubated with acetylation reagent (a 1:2 mixture of acetic anhydride and triethylamine) and anti-cGMP antibody. Mixtures were placed into the wells of a 96-well plate precoated with donkey anti-rabbit antibody and incubated at 4°C for 2 hours. Peroxidase-labeled cGMP conjugates were added to the mixture and further incubated at 4°C for 1 hour. After washing with 0.05% Tween 20, the peroxidase was developed with 3,3′,5′-tetramethylbenzidine and hydrogen peroxide and measured at 450 nm. cGMP concentrations were calculated and were referenced to the same solution provided by the manufacturer.

**Measurements of YC-1 levels in tumors.** YC-1 was extracted from tumor tissues using a 7:3 (v/v) mixture of diethyl ether and dichloromethane.
After evaporating the solvent, samples were dissolved in 80:20 (v/v) methanol/water, and then applied to a high-performance liquid chromatographer. The mobile phase gradient was composed of 10 mmol/L acetic acid in water (A) and 10 mmol/L acetic acid in acetonitrile (B). Elution was done at a flow rate of 0.25 mL/min sequentially in an 8:2 (v/v) mixture of solution A to B for 12 seconds, a 1:9 mix for 2.8 minutes, and an 8:2 mix for 10 minutes. A LUNA C18 analytic column (2 mm x 50 mm; Phenomenex, Torrance, CA) was used for reverse phase separation. YC-1 was quantified using an API-3000 Triple Quadrupole liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a turbo-ion spray interface (AB/MDS Sciex, Toronto, Ontario, Canada). Multiple reaction monitoring experiments in positive ionization mode were done using a dwell time of 300 ms per transition to detect ion pairs at m/z 305.2/197.2.

Statistical analysis. All data were analyzed using Microsoft Excel 2002 software. Results are expressed as means and SD. The Mann-Whitney U test (SPSS 10.0 for Windows software, Chicago, IL) was used to compare the cell populations in the S phase, protein levels, cell viabilities, and caspase-3 activities. Differences were considered to be statistically significant for P < 0.05. All statistical tests were two-sided.

Results

Effect of YC-1 on hypoxia-induced cell growth inhibition.

To examine whether YC-1 reverses hypoxia-induced proliferation inhibition, BrdUrd incorporation assays were done in Hep3B cells. Cell proliferation was noticeably inhibited under hypoxic conditions, as reported previously (8, 9). However, unexpectedly, YC-1 did not reverse the antiproliferative effect of hypoxia but rather augmented the inhibition of proliferation under hypoxic conditions (Fig. 1A). Moreover, the antiproliferative effect of YC-1 was also observed under normoxic conditions. Results were statistically analyzed and are plotted in Fig. 1B. YC-1 also inhibited HEK293 cell...
proliferation under normoxic conditions (Fig. 1C). These results suggest that YC-1 can inhibit HIF-1α without reverting proliferation inhibition through the direct inhibition of cell proliferation. Furthermore, it is suspected that antiproliferative activity in combination with anti-HIF activity probably reinforces the anticancer effect of YC-1.

S-phase arrest induced by YC-1. To clarify the mechanism of YC-1-induced proliferation inhibition, we examined the effect of YC-1 on the cell cycle. Cell populations in the G0-G1 and S phases were 60% and 24% in control Hep3B cells. However, after 24-hour incubation with 1 μmol/L YC-1, the S population was noticeably enhanced by 33%, whereas the G0-G1 population was decreased by 32% (Fig. 2A). The sub-G1 population was slightly increased in YC-1-treated cells. The S population increased in dose- and time-dependent manners (Fig. 2B and C, left). After YC-1 was removed from the medium, the S population gradually reduced to near control levels (Fig. 2C, right), suggesting that YC-1 has a reversible action on S-phase arrest. Moreover, YC-1 also induced S-phase arrest in other cell lines, namely HEK293 and Caki-1. Compared with Hep3B cells, HEK293 responded to YC-1 more sensitively, whereas Caki-1 was less sensitive (Fig. 2D, left). Interestingly, the G2-M population of HEK293 was markedly reduced after YC-1 treatment. The S-G2 transition seems to be completely blocked by YC-1 in HEK293, whereas this seems partial in Hep3B and Caki-1. As another index for S-phase arrest, we measured levels of cyclin D1, which is suppressed only in the S phase, and its expression was found to be suppressed by YC-1 in all three cell lines (Fig. 2D, right). In particular, cyclin D1 in HEK293 was almost completely suppressed by 1 μmol/L YC-1. This result further supports the high sensitivity of HEK293 to YC-1. Moreover, the ectopic expression of cyclin D1 failed to rescue HEK293 from YC-1-induced cell cycle arrest (data not shown), which suggest that cyclin D1 suppression may not be causative but rather the result of S-phase arrest. The S-phase arrest effect of YC-1 was confirmed in synchronized HEK293 cells. The cell cycle was restarted by washing out the hydroxyurea. In the absence of YC-1, G1-arrested cells entered the S and G2-M phases 12 hours after washout, and reentered the G1 phase 18 hours after washout. However, in the presence of YC-1, the majority of cells stayed in the S phase, and thus did not enter the G2-M phase (Supplementary Fig. S1).

YC-1 levels in tumors. To determine effective YC-1 concentrations, the Hep3B tumor-bearing mice were injected once with 30 mg/kg YC-1, and then YC-1 levels were measured in tumors. YC-1 was present in the range of 0.5 to 5 μmol/L in tumors 4 hours after injection (Fig. 3A), suggesting that it has anticancer activity in this concentration range. YC-1 is known to have three distinct biological actions—cGMP elevation (10), HIF-1α inhibition (2), and S-phase arrest induction (the present study). To determine which of these actions is responsible for its anticancer effect, we compared the YC-1 concentration ranges that cause these effects in Hep3B cells. Figure 3B showed that YC-1 inhibited HIF-1α expression in the range of 2 to 5 μmol/L. Interestingly, it was found that S-phase arrest and HIF-1α inhibition occurred in a similar concentration range to that observed in tumors, whereas cGMP elevation occurred at 10-fold higher concentrations (Fig. 3C). These results suggest that both S-phase arrest and HIF-1α inhibition may contribute to the anticancer effect of YC-1 but that cGMP elevation is unlikely to be responsible for its anticancer effect. Because S-phase arrest occurred concomitantly with HIF-1α inhibition, we considered the possible link between these events. However, knocking down of HIF-1α did not affect the cell cycle under either normoxic or hypoxic conditions (Fig. 3D), suggesting...
that the YC-1-induced S-phase arrest occurs independently of HIF-1α inhibition.

Delayed-onset apoptosis of cells arrested in the S phase.

To examine whether cell death is accompanied by S-phase arrest, we measured cell viabilities by using MTT assay. YC-1 induced delayed-onset cell death in a dose-dependent manner (Fig. 4A). Moreover, we confirmed this YC-1-induced apoptosis by measuring three different apoptotic markers, i.e., caspase-3 activity (Fig. 4B), PARP cleavage (Fig. 4C), and TUNEL staining (Fig. 4D). A caspase-3 inhibitor, Ac-DEVD-CHO, was used to confirm the caspase-dependent apoptosis in Fig. 4D. To see whether YC-1-induced apoptosis is preceded by S-phase arrest, we examined the time courses of S-phase arrest and apoptosis (indexed using the sub-G1 population) in synchronized cells. When cells were continuously incubated with hydroxyurea, most cells stayed in the G1 phase, regardless of YC-1 treatment; in this situation, hydroxyurea per se produced apoptosis, but YC-1 failed to augment this apoptosis (Fig. 5A). On the other hand, when cells were treated with YC-1 immediately after washing out the hydroxyurea, the majority of cells stayed in the S phase for 12 hours without showing distinct apoptosis. After 24 hours, dead cells gradually increased in the YC-1 group, but not significantly so in the control group (Fig. 5B). These results suggest that delayed-onset apoptosis induced by YC-1 is limited to cells arrested in the S phase.

Chk activation by YC-1. Chk1 and Chk2 are key mediators of intra-S-phase checkpoint signaling. These kinases promote the degradation of Cdc25A phosphatase, which in turn locks the S phase–promoting Cdk2 in its inactive phosphorylated form and prevents the loading of Cdc45 onto the replication origin (11, 12). Therefore, we tested the possibility that this checkpoint signaling is involved in YC-1-induced S-phase arrest. YC-1 activated both Chk1 and Chk2 in a dose-dependent manner (Fig. 6A). When Chk1 or Chk2 mRNA was knocked down using siRNA (Fig. 6B, top), YC-1-induced S-phase arrest was recovered (Fig. 6B, bottom). These results suggest that Chk activation by YC-1 causes S-phase arrest.

ATM/ATR–independent S-phase arrest. The most important cellular event provoking S-phase arrest is DNA breakage. In this case, ATM and ATR kinases function as either sensors of DNA breakage or transducers that activate Chk kinases (11, 13). To examine the involvement of ATM and ATR in YC-1-induced S-phase arrest, activated ATM was measured using anti-phospho-ATM antibody. Although ATM was slightly activated by YC-1, this activation was much lower than that by etoposide, a positive control of ATM activation (Fig. 6C). Because ATM activation was
not distinct, we reexamined the roles of ATM and ATR using siRNA (Fig. 6D, top). However, the S-phase arrest was not rescued in these knocked down cells (Fig. 6D, bottom), suggesting that either ATM or ATR do not initiate YC-1-induced S-phase arrest. We next addressed the mechanism by which YC-1 activates the intra-S-checkpoint. DNA fragmentation is a well-known stimulus for this checkpoint (11), but YC-1 did not induce DNA breaks in Comet assays (Supplementary Fig. S2A). H$_2$O$_2$, which provides a positive control for DNA breaks, produced many Comet tails in Hep3B cells but YC-1 did not. We also measured topoisomerase I and II activities because inhibition of these enzymes can also induce DNA breaks and single-strand DNA gaps (14, 15). However, YC-1 did not inhibit either enzyme (Supplementary Fig. S2B and C). Taken together, it does not seem that YC-1 directly injures DNA. However, how does YC-1 activate Chk kinases? This question remains to be answered.

Discussion

HIF-1a is believed to promote tumor growth and metastasis, and many efforts have been made to develop new anticancer agents based on HIF-1a inhibitors (16–19). However, this anticancer strategy has been rebutted by several research groups. Carmeliet et al. (20) reported that HIF-1a(-/-) tumors grew faster than HIF-1a(+/+) tumors, although tumor vessel formation was impaired. Mack et al. (21) also showed that VHL(-/-) tumors containing high HIF-1a levels grew slower than VHL(+/+) tumors despite the induction of HIF-1 target genes. Recently, Koshiji et al. (9) explained the mechanism underlying the tumor-inhibiting effects of HIF-1a. Summarizing, the proto-oncogene c-Myc represses p21 gene transcription, and HIF-1a displaces Myc binding from the p21 promoter by competing for its DNA-binding site, thereby inducing p21 expression and cell cycle arrest. Considering the apparently contrary actions of HIF-1a in cancer cells (i.e., that HIF-1a inhibition may suppress tumor growth and promote hypoxic tumor regrowth), it is uncertain how HIF-1a inhibitors work in tumors. Fortunately, the HIF-1a inhibitor YC-1 might not promote hypoxic tumor regrowth because it additionally promotes cell cycle arrest. Furthermore, because cell cycle arrest per se is also an important anticancer mechanism, YC-1 may induce the combined anticancer effects of HIF-1a inhibition and cell growth inhibition.

Here, we show that the tumor levels of YC-1 were <5 µmol/L in mice injected with 30 mg/kg YC-1. Because both HIF-1a inhibition and S-phase arrest were induced by YC-1 at <5 µmol/L, we believe that these effects are responsible for tumor growth inhibition by YC-1. However, a recent report (22) showed that YC-1 at >50 µmol/L induced G$_2$/M-phase arrest in hepatoma cell lines. This concentration is much higher (10 fold) than the tumor levels of YC-1 in vivo. We also examined the effect of YC-1 at 50 µmol/L, but G$_2$/M-phase arrest was not observed in our experimental settings. Instead, S-phase arrest and cell death were induced earlier by 50 µmol/L YC-1 than by 1 µmol/L YC-1. Thus, we believe that S-phase arrest is the main contributor to the anticancer effect of YC-1.

As for the mechanism by which YC-1 arrests the cell cycle, we first considered the possible link between HIF-1a inhibition and cell cycle arrest. However, because HIF-1a is negligibly expressed under normoxic conditions (Fig. 3B), HIF-1a inhibition by YC-1 is likely to have no effect on normoxic cells. Moreover, HIF-1a overexpression failed to rescue the cells from YC-1-induced cell cycle arrest (data not shown) and HIF-1a knockdown did not induce S-phase arrest (Fig. 3D). Therefore, we ruled out the possible effect of HIF-1a inhibition on cell cycle arrest. Second, we considered the possibility that p53 is a mediator of cell cycle arrest. However, the effect of YC-1 on the cell cycle was obvious in a p53-null cell line (Hep3B), and p53 expression was unaffected by YC-1 in HEK293 cells (data not shown). Thus, p53 is also unlikely to be involved in the event sequence triggered by YC-1. Finally, we found that the intra-S-phase checkpoint, which occurs via Chk1/2 activation, is involved in the action of YC-1, and that the cell cycle arrest induced by YC-1 is rescued by Chk1/2 siRNAs.

In general, the intra-S-phase checkpoint is activated by DNA damage encountered during the S phase or by damaged DNA that escapes the G$_2$/M checkpoint, and this activation leads to a replication stop (13). Moreover, damage sensors at this checkpoint encompass a large set of checkpoint proteins. When double-stranded DNA

![Figure 5](image)

**Figure 5.** YC-1-induced apoptosis is preceded by S-phase arrest. A, G$_2$/M-arrested cells are not killed by YC-1. HEK293 cells were arrested at the G$_2$/M phase by treatment with 1 mmol/L hydroxyurea for 24 hours and then treated with DMSO (Control) or 1 µmol/L YC-1 for various times. B, cells in the S phase undergo apoptosis. HEK293 cells were arrested at G$_2$/M by treating with 1 mmol/L hydroxyurea for 24 hours and allowed to reenter the cell cycle by washing out the hydroxyurea. The cells were then incubated in the presence of DMSO (Control) or 1 µmol/L YC-1 for different times, stained with propidium iodide, and subjected to cell cycle analysis. Apoptotic cell death was estimated to be represented by the sub-G$_1$ population. Points and columns, means of three experiments.
breaks are detected, ATM is activated, which activates Chk2. In contrast, when single-strand DNA breaks or gaps are detected, ATR is activated, which activates Chk1. Moreover, when double- and single-strand breaks are mixed in cells subjected to genotoxic insults, both ATM and ATR can be activated. In addition, Chk proteins are cross-activated by ATM and ATR, and thus both Chk1 and Chk2 are usually activated by genotoxic stress (13). Chk1 and Chk2 are structurally unrelated, but functionally overlapping serine/threonine protein kinases (23). Both share the upstream activators, ATM and ATR, and downstream effects including Cdc25A degradation. When phosphorylated by Chks, Cdc25A is degraded by the ubiquitin-proteasome system, and in the absence of Cdc25A, Cdk2-cyclin E/A complex remains in the inactive hyperphosphorylated form, which in turn inhibits the Cdc45-mediated initiation of replication and induces S-phase arrest (13). In the present study, we found that Chks are activated by YC-1 and that they mediate S-phase arrest and that this did not require ATM or ATR. Thus, we wonder whether it is possible that Chks are activated by kinases other than ATM or ATR. Recently, the presence of a new member of the ATM/ATR kinase family, ATX, was suggested (23). ATX has been reported to be activated by UV light and in response to double-strand breaks, and subsequently to activate Chks. Therefore, ATX could be a candidate upstream mediator of the YC-1 effect. DNA-dependent protein kinase (DNA-PK) is also a plausible upstream mediator. DNA-PK is a member of the phosphatidylinositol 3'-kinase-like kinase family and is activated by ionizing radiation or UV light and mediates cell cycle arrest and apoptosis (24). Recently, Li and Stern (25) showed that Chk2 is phosphorylated by purified DNA-PK in vitro and that the down-regulation of DNA-PK attenuates Chk2 phosphorylation in irradiated cells. However, we ruled out the possibility that DNA-PK mediates YC-1-induced S-phase arrest for two reasons, namely, because the knocking out of DNA-PK disrupts apoptosis but not cell cycle arrest (26) and because the biological effects of DNA-PK tend to depend on phospho-regulation (27), whereas YC-1 induces cell cycle arrest and the action of YC-1 is independent of phospho-regulation. Therefore, the upstream activator of YC-1-induced Chk activation and S-phase arrest remains unidentified.

YC-1 was developed as an activator of soluble guanylyl cyclase (28). It increases the catalytic rate of the enzyme and sensitizes enzyme activation by nitric oxide or carbon monoxide (29). In terms of its pharmacologic actions, YC-1 prevents intravascular thrombus formation by inhibiting platelet aggregation (30), inhibits its vascular spasm by relaxing vascular smooth muscle (31), and strengthens penile erection by relaxing corpus cavernosal smooth muscle (32). On the other hand, if YC-1 is used for cancer therapy, these pharmacologic actions could have untoward effects like increasing bleeding time and hypotension. To develop YC-1 as an anticancer agent, these untoward effects should be carefully considered. In our opinion, these effects may not be critical enough to restrict the clinical use of YC-1 because they are clinically manageable. In addition, we found that both the anti-HIF and cell cycle arrest effects of YC-1 occur at ~10-fold lower concentrations than its cGMP elevation effect. Thus, it is possible that YC-1 at the dosages used for cancer chemotherapy does not produce the cGMP-mediated side effects. Previously, no serious toxicity was observed in nude mice treated with YC-1 over a 2-week period (3). Therefore, we consider that YC-1 is worth investigating further in terms of its clinical applications in cancer therapy.

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