PROSPECTS
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Recent Agents Targeting HIF-1α for Cancer Therapy

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ABSTRACT

The discovery of hypoxia-inducible factor-1 (HIF-1) has led to an increasing understanding of the mechanism of tumor hypoxia in the past two decades. As a key transcriptional regulator, HIF-1 plays a central role in the adaptation of tumor cells to hypoxia by activating the transcription of targeting genes, which regulate several biological processes including angiogenesis, cell proliferation, survival, glucose metabolism and migration. The inhibitors of HIF-1 in cancer have provided us a new clue for the targeting cancer therapy. This review will introduce the general knowledge of the biology characteristic of HIF-1 and mechanism of O_2-dependent regulation. Moreover, a number of chemical inhibitors plus protein and nucleic acid inhibitors are included and classified mainly based on their different mechanism of inhibiting action. We also prefer to discuss the advantages of protein and nucleic acid inhibitors compared with chemical inhibitors. J. Cell. Biochem. 114: 498–509, 2013.

KEY WORDS: HIF-1α; CHEMICAL INHIBITORS; PROTEIN AND NUCLEIC ACID INHIBITORS; VHH; CANCER; TARGETED THERAPY

The transcription factor hypoxia-inducible factor-1 (HIF-1) was first isolated by Semenza et al. [1997] from the extract of nucleus in hypoxic cells [Wang and Semenza, 1993]. Further studies have demonstrated that, HIF-1 is an oxygen-regulated transcriptional activator and plays central roles in mammalian development, physiology, and disease pathogenesis. Today, a large body of data has indicated that HIF-1 regulates the expression levels of more than 200 kinds of target hypoxia responsive genes. At least 70 putative hypoxia-inducible genes have been found to be directly modulated by HIF-1 and, with the studies going deeper, the number is still increasing [Semenza, 2007; Takenaga, 2011]. After about two decades of attempts, many HIF-1 inhibitors have been identified, the paper will review the chemical inhibitors (Table I), but also protein and nucleic acid agents targeting HIF-1α (Table II), and state advantages of antibody inhibitors than chemical inhibitors, hope to provide some useful insights for the further clinical development.

STRUCTURE OF HIF-1

HIF-1 (Fig. 1) is a heterodimer consisting of two subunits, a hypoxically inducible subunit HIF-1α and a constitutively expressed subunit HIF-1β, both subunits are members of the basic helix-loop-helix-Per-ARNT-Sim (bHLH–PAS) protein family [Jiang et al., 1996].

From N-terminal to C-terminal, HIF-1α contains bHLH and PAS domains (PAS-A, and PAS-B) that are required for dimerization and DNA binding, followed by the oxygen-dependent degradation domain (ODD) which confers oxygen-dependent regulation and two independent transcriptional activation domains, N- and C-terminal transactivation domains (N-TAD, and C-TAD), which are separated by an inhibitory domain (ID) [Verheul et al., 2008]. Additionally, HIF-1α contains two nuclear localization signals: N-NLS, and C-NLS. The C-terminal NLS is crucial in the nuclear import of HIF-1α, whereas the N-terminal one seems to be less important [Wang et al., 1995]. With the similar structure, the subunit of HIF-1β is critically involved in a range of transcriptional systems, and is indispensable for HIF-1 DNA binding and transactivation [Semenza et al., 1997].

REGULATION OF HIF-1α

Cells transduce decreased O_2 concentration into increased HIF-1 activity via O_2-dependent post-translation modification (Fig. 2). Under normoxic conditions, HIF-1-prolyl hydroxylases 1–3 (PHD1–3) hydroxylate the prolyl residues at amino acid 402, and 564, which

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TABLE I. Chemical Inhibitors Targeting HIF-1

Inhibition mechanisms		Agents	Current status	References
Transcriptional activity/ DNA binding	Regulate on multiple levels	YC-1 and analogs	Preclinical	Chun et al. [2001, 2004], Li et al. [2008], Sun et al. [2007]
	Inhibiting thioredoxin-1	PX-12	Phase II	Welsh et al. [2003]
		Pleurotin	Preclinical	
	Inhibiting thioredoxin-1 and DNA binding	AJM290/AW464	Not in clinical use	Jones and Harris [2006], Jones et al. [2006]
	Inhibiting HRE and DNA binding	Echinomycin (NSC-13502)	Suspended	Kong [2005], Vlaminck et al. [2007]
	Disrupting binding of HIF-1–HRE	Polyamides	Not in clinical use	Jacobs and Dervan [2009]
	Inhibiting HIF-1α gene transcription	Flavopiridol (alvocidib)	Phase III	Blagosklonny [2004], Newcomb et al. [2005]
	Inhibiting p300 recruitment	Chetomin	Preclinical	Kung et al. [2004]
		Bortezomib (Velcade, PS-341)	Approved	Shin et al. [2008]
	Inhibiting FIH-1, and the p300 recruitment	Amphotericin B	Approved	Yeo et al. [2006]
HIF-1α translation	Inhibiting PI3K	LY294002 and Wortmannin	Not in clinical use	Jiang et al. [2001]
	Blocking mTOR	Rapamycin, Everolimus and CCI-779	Approved	Cejka et al. [2008], Hudson et al. [2002], Wan et al. [2006]
	Blocking mTOR/p70S6K/4E-BP1 pathway	Silibinin	Approved	Garcia-Maceira and Mateo [2009]
	Microtubule depolymerization	2ME2 and ENMD-1198	Phase II/Phase I	LaVallee et al. [2008], Mabjeesh et al. [2003]
	Regulate on multiple levels, specific unknown	PX-478	Phase I	Welsh et al. [2004]
	Inhibit HIF-1α mRNA translation	2-phenethyl isothiocyanate	Phase II	Wang et al. [2009]
HIF-1α degradation	Inhibiting Hsp90	GA and analogs	Phase II	Isaacs et al. [2002], Mabjeesh et al. [2002]
		Radicicol and its derivants	Not in clinical use	Hur et al. [2002], Kurebayashi et al. [2001]
		Apigenin	Phase II	Fang et al. [2005]
	Inhibiting HDAC (Histone deacetylase)	Trichostatin A (TSA)	Phase I	Yang et al. [2006]
		LAQ824	Not in clinical use	Qian et al. [2006]
		SAHA and FK228	Approved	Mie et al. [2003], Shankar et al. [2009]
	Increasing HIF-1α and pVHL interaction	Wondonin	Not in clinical use	Jun et al. [2007]
	Blocking signaling pathways	Green tea extract and EGCG	Not in clinical use	Zhang et al. [2006]
	Inhibiting ERK 1/2 and AKT activation	Resveratrol	Phase II	Park et al. [2007], Zhang et al. [2005]

is required for the binding of the von Hippel–Lindau (VHL) tumor-suppressor protein. VHL binding is also promoted by acetylation of lysine residue 532 by the ARD1 acetyltransferase, then poly-ubiquitinized, resulting in the degradation of HIF-1α by the 26S proteasome. Oxygen-dependent hydroxylation of asparagine residue 803 in HIF-1α by the enzyme FIH-1 (factor inhibiting HIF-1) blocks the binding of p300 and CBP to HIF-1α and therefore inhibits HIF-1-mediated gene transcription. Under hypoxic conditions, HIF-1α is not hydroxylated because the major substrate, O_2 is not available. The unmodified protein escapes the VHL-binding, ubiquitination, and degradation, and then dimerizes HIF-1 and stimulates the transcription of its target genes [Semenza, 2003].

TABLE II. Protein and Nucleic Acid Inhibitors Targeting HIF-1α

Inhibition mechanisms		Agents	References
HIF-1α stability	Attenuate HIF-1α stabilization possibly through zinc deprivation	Metallothioneins (MTs)	Devisscher et al. [2011]
	Cetuximab reduced the cellular level of HIF-1α in the presence of a proteasome inhibitor, lactacystin, indicating that cetuximab acts mainly at the level of protein synthesis	Cetuximab	Luwor et al. [2005]
Transcriptional activity	Implicated in the induction of HIF-1α translation, has antiangiogenic effect by inhibiting HIF-1 dependent induction of VEGF	Herceptin	Koukourakis et al. [2003]
mRNA and protein expression	RNA interference is the process of sequence specific post-transcriptional gene silencing, small Interfering RNA (siRNA) have been demonstrated to have stronger suppressive effects, in order to inhibit HIF-1α expression	Small Interfering RNA (siRNA)	Mizuno et al. [2006]
	RX-0047 is a 20-mer phosphorothioate antisense oligonucleotide (ASO) that is a potent inhibitor of HIF-1a. RX-0047 inhibits HIF-1a by reducing expression of its mRNA and protein. The results have been tested by experiment in vivo and in vitro	RX-0047	Dikmen et al. [2008]
Dimerization of HIF-1α and HIF-1β	Single-domain llama antibodies (VHH) directed against the HIF-1α oxygen dependent degradation domain (ODDD) which encompass the N-terminal transactivation domain (N-TAD), the combination of them can prevent the dimerization of HIF-1α and HIF-1β; hetero-bivalent VHH markedly increased the binding affinity for HIF-1α, to be novel agents for cancer therapy	Anti-HIF-1α VHH and Hetero-bivalent VHH nanobodies (AG1-5)	Groot et al. [2006, 2008]
	Anti-HIF-1α VHH nanobodies (AHPC) screened by our laboratory is able to recognize HIF-1α within the PAS domain and combine with it specifically in order to prevent the dimerization of HIF-1α and HIF-1β; the further characteristic of it is still under research	Anti-HIF-1α VHH nanobodies (AHPC)	Unpublished

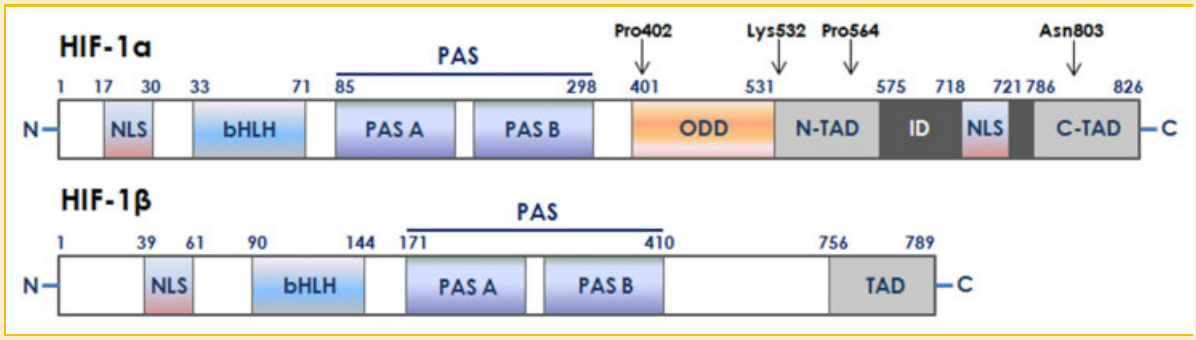

Fig. 1. Schematic representation of structure of HIF-1α and HIF-1β. The figure has been adapted, with some modifications, from Li and Ye [2010]. Numbers indicate the amino-acid residues of each domain or special amino sites. From N-terminal, HIF-1α contains nuclear localization signals (NLS), a basic helix-loop-helix (bHLH) domain and two Per-ARNT-Sim homology domains (PAS-A and PAS-B). N- and C-terminal transactivation domains (N-TAD and C-TAD) are separated by an inhibitory domain (ID).

As stated above, O_2 concentration can regulate HIF-1α protein levels, however, the pVHL pathway is not the only pathway controlling levels of HIF-1α protein (as shown in Fig. 3). HIF-1α translation can be stimulated by growth factors that activate the phosphatidylinositol 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways. In the MAPK pathway [Sang et al., 2003], the extracellular signal-regulated kinase (ERK) is activated by the upstream MAP/ERK kinase (MEK). ERK, in turn, activates MNK. ERK and mTOR phosphorylate p70 S6 kinase (S6K) and the eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1). Binding of 4E-BP1 to eIF-4E inactivates the latter, inhibiting cap-dependent mRNA translation. Phosphorylation of 4E-BP1 prevents its binding to eIF-4E. MNK phosphorylates eIF-4E and stimulates its activity directly. Thus, the inhibition of the regulatory factors, such as PI3K, mTOR, MNK and so on [Zhong et al., 2000], can inhibit the translation of HIF-1α mRNA, in order to find novel agents for cancer treatment [Alvarez-Tejado et al., 2001]. As shown in Figure 3, antibodies that can bind to HIF-1α block the binding of HIF-1α, and HIF-1β in order to prevent the forming of HIF-1, then inactivates the transcriptional activity.

CHEMICAL INHIBITORS

It is well know that, the levels of HIF-1 are mainly controlled by the regulation of HIF-1α. Many chemical inhibitors of HIF-1α have been developed and the increasing agents are constantly being reported. According to their mechanism of inhibiting action, chemical inhibitors of HIF-1α could be divided in agents that modulate:

(1) HIF-1α transcription and transcriptional activity;
(2) HIF-1α protein translation;
(3) HIF-1α DNA binding;
(4) HIF-1α protein degradation.

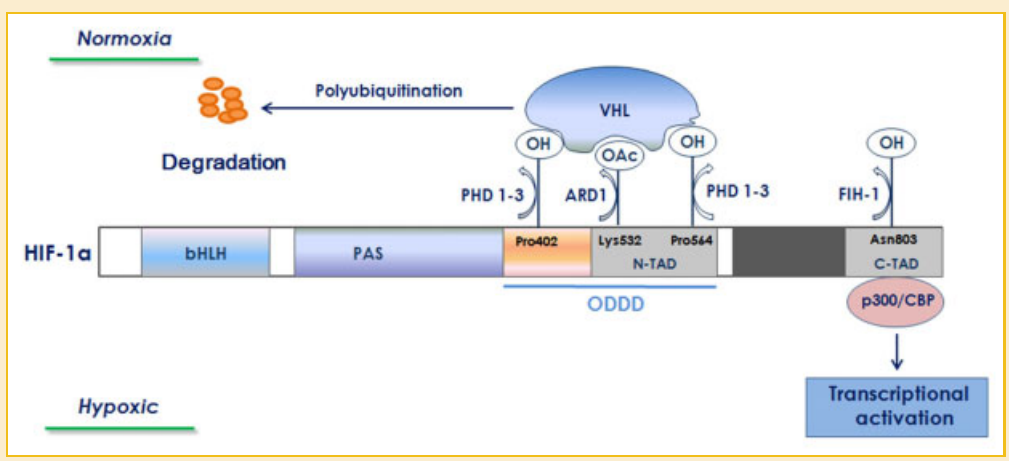

Fig. 2. Schematic representation of oxygen-dependent regulation of HIF-1α activity. The figure has been adapted, with some modifications, from Semenza [2003]. O_2 regulates the rate at which HIF-1α protein is degraded. In normoxic conditions, O_2-dependent hydroxylation of proline residues 402 and 564 in HIF-1α by the enzymes prolylhydroxylase-domain protein (PHD) 1–3 is required for the binding of the von Hippel-Lindau (VHL) tumor-suppressor protein. VHL binding is also promoted by acetylation of lysine residue 532 by the ARD1 acetyltransferase. Oxygen-dependent hydroxylation of asparagine residue 803 in HIF-1α by the enzyme FIH-1 (factor inhibiting HIF-1) blocks the binding of p300 and CBP to HIF-1α and therefore inhibits HIF-1-mediated gene transcription. Under hypoxic conditions, VHL cannot bind to HIF-1α, by contrast, p300 and CBP can bind to HIF-1α, allowing transcriptional activation or HIF-1 target genes.

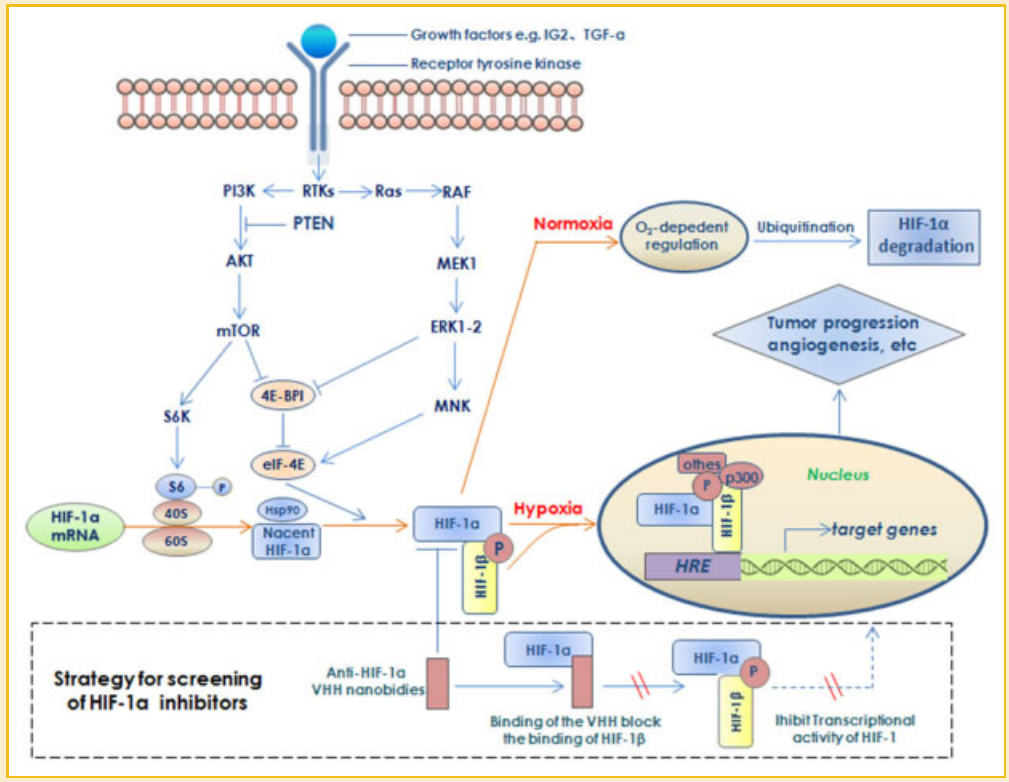

Fig. 3. Important pathways regulating HIF-1α translation and proposed mechanisms of action of HIF-1 inhibitors. In the MAPK pathway, the extracellular signal-regulated kinase (ERK) is activated by the upstream MAP/ERK kinase (MEK). Then ERK activates MNK. ERK and mTOR phosphorylate p70 S6 kinase (S6K) and the Eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1). Binding of 4E-BP1 to eIF-4E inactivates the latter, inhibiting cap-dependent mRNA translation. Phosphorylation of 4E-BP1 prevents its binding to eIF-4E. MNK phosphorylates eIF-4E and stimulates its activity directly. VHH nanobodies that can bind to HIF-1α block the binding of HIF-1α and HIF-1β in order to prevent the forming of HIF-1, then inactivates the transcriptional activity.

INHIBITORS OF HIF-1α TRANSCRIPTION, TRANSCRIPTIONAL ACTIVITY, OR DNA BINDING

Under hypoxic conditions, the levels of HIF-1 protein depend on the levels of HIF-1α mRNA, thus, it is sure that chemical inhibitors of HIF-1α transcription could affect the rate of HIF-1α translation, as a consequence, decrease the HIF-1α transcriptional activity. Inhibition of HIF-1 binding to the hypoxia responsive element (HRE), a step required for induction of transcription of target genes, is also a potential mechanism by which small molecules may inhibit HIF-1α activity [Lee et al., 2009].

YC-1 and analogs. YC-1 was originally used as a pharmacological tool to activate soluble guanylate cyclase and to increase cyclic GMP levels, and then YC-1 was limited to be an inhibitor of platelet aggregation and vascular concentration [Chun et al., 2004]. However, recent reports suggested that YC-1 could be developed as a new class of anticancer agent for its inhibition on HIF-1α activity and the expression of HIF-1-target genes, YC-1 regulated HIF-1α activity on multiple levels [Chun et al., 2001]. It was confirmed that YC-1 prevented p300 recruitment by C-TAD in mammalian two-hybrid and coimmunoprecipitation assays. In cancer cells, YC-1 inhibited HIF-1α via stimulation of FIH dependent p300 dissociation from HIF-1α [Li et al., 2008]. However, YC-1 was also found to suppress the PI3K/AKT/mTOR/4E-BP pathway, which severed to regulate HIF-1α expression at the translation step. We demonstrated that YC-1 inhibited hypoxia-induced activation of nuclear factor (NF)-kappaB, a downstream target of AKT [Sun et al., 2007]. Recently, they also found two novel YC-1 analogs: one is an inhibitory effect on either HIF-1 or HIF-2 activity; the other is an anti-proliferative effect on cancer cells by arresting cell cycling and leading to apoptosis.

PX-12 and pleurotin. Thioredoxin-1 (Trx-1) is a small redox protein over expressed in a number of human primary tumors [Welsh et al., 2002]. As the inhibitors of Trx-1, PX-12 (1-methylpropyl 2-imidazolyl disulfide) and pleurotin were originally found to decrease HIF-1α protein levels, HIF-1 transactivation and the expression of downstream target genes in human cancer cells [Welsh et al., 2003]. The mechanism of the decrease in HIF-1α protein by these Trx-1 inhibitors is unknown. Recently, two inhibitors of Trx-1, AJM290 [Jones and Harris, 2006] and AW464 [Jones et al., 2006], were found to paradoxically increase HIF-1α protein levels, thus, inhibit HIF-1 DNA binding and transcriptional activity.

Echinomycin (NSC-13502) and polyamide. Some inhibitors of HIF-1α do not affect the mRNA levels or protein levels but inhibit the binding of HIF-1α to target DNA and prevent the activation of transcription. Echinomycin (NSC-13502), originally isolated from *Streptomyces echinatus*, is a cyclic peptide of the quinoxaline antibiotic family. Previous studies have demonstrated that, it

specifically binds to the core of the HIF-1α recognition sequence and inhibits binding of HIF-1 to the HRE sequence [Kong, 2005]. Moreover, it is demonstrated that echinomycin induces an increase in HIF-1 activity under normoxic conditions, parallel to an increase in the expression of HIF-1 target genes. This effect is caused by an increase in HIF-1α protein levels, resulting from an increase in the transcription of the HIF-1α gene in the presence of a low concentration of echinomycin [Vlaminck et al., 2007].

Polyamides are a new class of programmable sequence-specific DNA-binding oligomers. In particular, sequence-specific synthetic polyamides were designed to inhibit binding of the HIF-1α heterodimer to its cognate DNA sequence. This HRE-targeted polyamide inhibited binding of HIF-1 to HRE, and blocked hypoxic induction of VEGF mRNA expression and protein in HeLa cells [Jacobs and Dervan, 2009].

Flavopiridol (alvocidib). Flavopirdol is a kind of flavones synthetically derived from an alkaloid isolated from the leaves and stems of *Amoora robituka*, and *Dysoxylum binectariferum* (plants indigenous to India). As a potent inhibitor of global transcription, flavopirdol can down-regulate numerous protein, cell cycle arrest and apoptosis [Newcomb et al., 2005]. As an inhibitor of transcription with a unique mechanism of action, flavopiridol has been under clinical trial. By inhibiting CDK-9 and also -8, flavopiridol inhibits transcription. CDK-9 (in complex with cyclin T) is the transcriptional elongation factor P-TEFb, which does not participate in cell cycle regulation. Studies have shown the mechanisms of action of flavopiridol, inhibition of transcription by inhibiting P-TEFb [Blagosklonny, 2004].

Inhibitors of p300. Chetomin, one member of the Epidithiodiketopiperazines family (ETP), was obtained from fungus *Chaetominum* species and originally was found with antimicrobial activity. Chetomin inhibits the HIF-1 transcriptional activity by a mechanism of disrupting the global fold of the cysteine–histidine-rich domain 1 (CH1) domain of p300 and thereby preventing interaction of p300 and HIF-1α, leading to tumor growth inhibition. The xenograft model experiments have demonstrated that, systemic administration of chetomin inhibited hypoxia-inducible transcription within tumors and tumor growth [Kung et al., 2004].

Bortezomib (Velcade, PS-341), a proteasome inhibitor, directly induces tumor cell death and has been reported to inhibit tumor adaptation to hypoxia by functionally inhibiting of HIF-1α. Bortezomib, which targets HIF-1α C-terminal transactivation domain (C-TAD) indirectly inhibits p300 recruitment by the mechanism of enhance the interaction between FIH-1 and C-TAD [Shin et al., 2008]. In May 2003, FDA approved bortezomib in the US for use in multiple myeloma based on the good results of clinical trials.

Amphotericin B is widely used for treating severe systemic fungal infections, however, recent studies have demonstrated that Amphotericin B inactivated the transcriptional activity of HIF-1α, but did not affect the expression or localization of HIF-1 subunits. The mechanism involved repression of C-TAD of HIF-1α, furthermore, inhibiting of the p300 recruitment by stimulating C-TAD and FIH interaction [Yeo et al., 2006].

INHIBITORS OF HIF-1α PROTEIN TRANSLATION

Inhibitors blocking PI3K/AKT/mTOR signal pathway. During the process of HIF-1α protein translation, PI3K/AKT/mTOR signal pathway plays an important role, whereas, the inhibition of the pathway will be effective to decrease the HIF-1α protein levels, in order to find novel agents for the cancer treatment. According to the previous studies, in the prostate carcinoma-derived cell lines PC-3 and DU145, the PI3K-specific inhibitors LY294002 and wortmannin inhibited the expression of HIF-1α protein in a dose-dependent manner [Jiang et al., 2001]. Rapamycin [Hudson et al., 2002], which had been accepted for the clinical use, and its chemical derivatives, CCI-779 [Wan et al., 2006] and Rad001 [Cejka et al., 2008], specially block mTOR, then inhibit PI3K signal pathway, and have been demonstrated to reduce cellular levels of HIF-1α protein.

Silibinin, as a nontoxic flavonoid, was found to inhibit hypoxia-induced HIF-1α accumulation and HIF-1 transcriptional activity in human cervical (HeLa) and hepatoma (Hep3B) cells. Rather, it is found that suppression of HIF-1α accumulation by silibinin correlated with strong dephosphorylation of mammalian target of rapamycin (mTOR) and its effectors ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), the pathway is well known to regulate HIF-1α expression at the translation level [Garcia-Maceira and Mateo, 2009]. Silibinin was also found to be a potent inhibitor of cell proliferation.

2-Methoxyestradiol (2ME2) and analogs. 2-methoxyestradiol (2ME2) and its analogs are a group of the most promising HIF-1α inhibitors for the cancer treatment and currently in a state of clinical trials. Recently, 2ME2 was shown to inhibit HIF-1α translation and its nuclear translocation, downstream of microtubule disruption, which was associated with antiangiogenic activity [Mabjeesh et al., 2003]. In order to improve the pharmacokinetic and inhibitory characteristic, some analogs of 2ME2 are derived. The analogs all show inhibitory effect of HIF-1α levels, whereas enhanced metabolic stability. Among them, ENMD-1198 [LaVallee et al., 2008] was selected as the representative compound for evaluation in a Phase I clinical trial for safety, tolerability, pharmacokinetics, and clinical benefit in advanced cancer patients. As well as PX-478 (S-2-amino-3-[40-*N*,*N*-bis(2-chloroethyl)amino] phenyl propionic acid N-oxide dihydrochloride), a novel agent derived from melphalan by oxidation of the nitrogen mustard, studies of the mechanism of PX-478 showed that it inhibited HIF-1α at multiple levels, including decreasing HIF-1α mRNA levels, reducing HIF-1α translation and promoting degradation of HIF-1α [Welsh et al., 2004].

2-Phenethyl isothiocyanate. Phenethyl isothiocyanate (PEITC), a natural dietary isothiocyanate, have been demonstrated to be an effective inhibitor of HIF-1α. Studies found that decreased expression of HIF-1α in PEITC treated cells was not associated with changes in the levels of HIF-1α mRNA suggesting that PEITC may inhibit HIF-1α activity by decreasing translation of the HIF-1α mRNA. Consistent with this, PEITC decreased phosphorylation of the translation regulator 4E-BP1 [Wang et al., 2009].

AGENTS TARGETED TO HIF-1α PROTEIN DEGRADATION

Inhibitors of Hsp90. Hsp90 is a kind of molecular chaperone that interacts with HIF-1α and is required for HIF-1 protein stability.

Geldanamycin (GA) is a naturally occurring benzoquinone ansamycin antibiotic that interferes with Hsp90 function by competing with its ATP binding site. Through the tumor inhibition experiments, GA was found to induce ubiquitination and proteasome-mediated degradation of HIF-1α in a VHL-independent fashion [Isaacs et al., 2002]. 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), the analogs of GA, are now in Phase II, and Phase I clinical trial respectively [Mabjeesh et al., 2002]. Other inhibitors of Hsp90 have also been implicated with the same mechanism, including radicicol [Hur et al., 2002] and its derivative KF58333 [Kurebayashi et al., 2001], the farnesyltransferase inhibitor apigenin [Fang et al., 2005].

Inhibitors of histone deacetylase (HDAC). HDAC inhibitors promote HIF-1α degradation by the mechanism of upregulating p53 and VHL. Moreover, it is demonstrated that HIF-acetylation is also important for its activity [Lim et al., 2010]. Trichostatin A (TSA) is an organic compound that serves as an antifungal antibiotic. In correlated studies, TSA showed a dose- and time-dependent inhibition of HIF-1α levels. TSA-mediated repression of HIF-1α is independent of VHL and p53, but is mediated by the proteasome system [Yang et al., 2006]. It was reported that treatment of the VHL-deficient human renal cell carcinoma cell line UMRC2 with the hydroxamic HDAC inhibitor LAQ824 resulted in a dose-dependent inhibition of HIF-1α protein via a VHL-independent mechanism and reduction of HIF-1α transcriptional activity, associated with HIF-1α acetylation and polyubiquitination [Qian et al., 2006]. In vivo treatment with SAHA followed by tumor necrosis factor-related apoptosisinducing ligand (TRAIL), which resulted in the down-regulation of NF-κB and its gene products, involving VEGF and HIF-1α, and upregulation of a series of endogenous tumor suppressors, leading to inhibition of tumor progression, angiogenesis, and metastasis [Shankar et al., 2009]. Then, the inhibitor FK228 has been found to inhibit HIF-1α by a transcription-dependent mechanism [Mie et al., 2003].

Inhibit HIF-1α by other mechanisms. Wondonin, a bis(dihydroxystyryl) imidazole purified from an association of the sponges, significantly decreased hypoxia-induced HIF-1α protein and VEGF expression and inhibited angiogenesis in vitro, and in vivo by a mechanism of enhancing the interaction between HIF-1α, and pVHL [Jun et al., 2007].

Existing knowledge has proved that green tea extract and its major polyphenol component (−)-epigallocatechin-3-gallate (EGCG) could inhibit HIF-1 protein accumulation and VEGF expression. The detailed mechanism involves blocking the related signaling pathways and enhancing HIF-1α protein degradation [Zhang et al., 2006].

Resveratrol, a natural product commonly found in grapes and various other fruits and flavonoids, has been demonstrated to inhibit HIF-1α protein level by inhibiting ERK 1/2 and AKT activation, and now in Phase II clinical trial [Zhang et al., 2005; Park et al., 2007].

PROTEIN AND NUCLEIC ACID INHIBITORS

At present, it seems that more and more researchers have focused their attention on the targeted therapy of cancer. However, the common deficiencies of most HIF-1α chemical inhibitors described above is the lack of specificity, which indicates that they inhibit multiple targets, so the development of specific inhibitors is of great urgency. The production of monoclonal antibodies (mAbs) has promoted the progress of antibody engineering, and then the small size engineering antibodies came out, such as the single chain variable fragments (scFv). In 1993, heavy-chain antibodies (HCAbs) lacking light chains occur naturally in camelid [Hamers-Casterman et al., 1993] was found and given new hope for cancer therapy. So far, some specific inhibitors have been discussed and the paper will review the inhibition mechanism of them in order to shine new light on clinical application.

Protein inhibitor. The positive-feedback mechanism between HIF-1α, and metallothioneins (MTs) has been identified in different diseases. Devisscher et al. [2011] investigated the interaction between HIF-1α and MTs in colonic epithelial cells and demonstrated the HIF-1α-mediated down-regulation of MTs in colonic epithelial cells. MTs were able to attenuate HIF-1α stabilization possibly through zinc deprivation. So agents targeting MTs and enhancing the protective effects of HIF-1α may represent future therapeutic opportunities.

Monoclonal antibody inhibitors. Cetuximab is a chimeric (mouse/human) monoclonal antibody, an epidermal growth factor receptor (EGFR) inhibitor, and initially prepared for treatment of metastatic colorectal cancer. Recently, researchers have furthered the study by demonstrating that cetuximab reduces levels of HIF-1α. Cetuximab reduced the cellular level of HIF-1α in the presence of a proteasome inhibitor, lactacystin, indicating that cetuximab acts mainly at the level of protein synthesis. The reduction of HIF-1α in response to cetuximab treatment was accompanied by transcriptional inhibition of VEGF expression. The results indicated that the previously demonstrated inhibition of VEGF by cetuximab occurs at the level of transcription in response to a reduced level of HIF-1α [Luwor et al., 2005].

Herceptin, a humanized monoclonal antibody, had been approved by FDA for the therapy of Her2/Neu positive breast cancer by interfering with the HER2/neu receptor. Her2/Neu signaling has been implicated in the induction of HIF-1α translation in breast cancer cells, then herceptin may show an antiangiogenic effect, at least partly, by inhibiting HIF-1 dependent induction of VEGF [Koukourakis et al., 2003], the clinical study also provided evidence.

Nucleic acid inhibitor. Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20–25 nucleotides in length, that play a variety of roles in cells. These siRNAs have been demonstrated to have stronger suppressive effects than antisense oligonucleotides. Furthermore, recent studies have demonstrated that siRNA expression vectors suppress the expression of endogenous genes and exhibit more persistent inhibition compared to synthetic siRNAs. Mizuno et al. [2006] has found that siRNA expression vectors can inhibit the expression of HIF-1α and of those genes induced by HIF-1α, thus effectively suppressing the in vitro and in vivo growth of selected hepatobiliary tumors.

RX-0047, as a 20-mer phosphorothioate antisense oligonucleotide (ASO), is a potent inhibitor of HIF-1a. RX-0047 directly inhibits HIF-1a by reducing expression of its mRNA and protein. The inhibiting effects of RX-0047 had been analyzed in various human

cancer cell lines, including the lung cancer cells (A549), pancreatic cancer cells (Panc1), prostate cancer cells (PC-3) and breast cancer cells (MDA-MB-231 andHME50-T) [Dikmen et al., 2008]. It was demonstrated that RX-0047 is cytotoxic in vitro against various cancer cell types in nanomolar concentrations. In vivo data showed that RX-0047 inhibited tumor formation of the PC-3-Luc cells in flank tumor model and effectively prevented lung metastasis in nude mice with intravenously introduced cells.

Nanobody inhibitors. New kinds of antibodies, llama heavy-chain antibody, which are also referred to VHH or nanobodies are stable at high temperatures and can bind antigen in high salt concentrations. The characteristic of small molecular can make nanobodies go through the membrane structure, cripple intracellular viral replication, block enzymatic activity, pass the blood–brain barrier and can be used for various immunological applications like classical antibodies.

Nanobodies can specifically target and inhibit HIF-1 expression for several reasons. The first reason is the sensitive screening method. The majority of HIF-1 chemical inhibitors identified so far are the result of either cell-based screens or empirical discoveries during evaluation of HIF-1 activity in cultured cancer cell lines. However, none of the present available inhibitors appears to disrupt the HIF-1 pathway as their exclusive target. Therefore, design of more specific HIF-1 targeting agents is likely to become the focus of future research efforts. It is also clear that the success of such studies will critically depend on the availability of sensitive screening methods [Belozerov and Van Meir, 2005; Xia et al., 2012]. Phage display technology came out about 20 years ago, the applications and development of it are only beginning to be explored. Exploitation of phage display technology will lead to the production of a broad range of binders with predefined specificities [Azzazy and Highsmith, 2002; Yip and Ward, 2002]. Nanobodies against essential domain of HIF-1 protein are screened based on phage display technology and can target HIF-1 pathways specially. Second reason is based on its unique structure. A better antigen binding capacity of nanobodies was observed than conventional antibodies. Their variable domains can bind a wide range of antigens, ranging from small molecules, such as haptens [Spinelli et al., 2000] and peptides [Rahbarizadeh et al., 2004], to large antigens, such as proteins [Spinelli et al., 1996] or viruses [Ledeboer et al., 2002]. Third reason is the inhibiting mechanism of nanobodies. Nanobodies are screed against essential domain of HIF-1 pathways, for example, the ODD domain, and PAS domain. They are necessary for synthesis, translation, transcription and oxygen-dependent regulation. Nanobodies recognize the domain and specially bind with it, in order to block the synthesis, speed the degradation of HIF-1 protein or affect transcriptional activities of targeted genes. Many researchers have focused their attention on the field.

Groot et al. [2006] have identified a set of VHH against both human and mouse HIF-1α. These VHH were mapped to epitopes within the oxygen-dependent degradation domain (ODDD), which contains both target sites of prolyl hydroxylation, essential for pVHL-dependent proteasomal degradation of the HIF-1α protein, the combination of them can also prevent the dimerization of HIF-1α, and HIF-1β. These anti-HIF-1α VHH were engineered into higher affinity bivalent VHH [Groot et al., 2008].

In our laboratory, the nanobodies specific to HIF-1α of pancreatic cancer were isolated from a naive camelidae VHH library. The nanobody is against of Per-Arnt-Sim-B domain (PAS-B) of HIF-1α. PAS-B is mainly responsible for the dimerization of HIF-1α, and HIF-1β and represents a novel drug target [To et al., 2006]. Anti-HIF-1α VHH nanobodies (AHPC) is able to recognize HIF-1α within the PAS-B domain and combine with it specifically in order to prevent the dimerization of HIF-1α, and HIF-1β, reduces the level of HIF-1 and blocks tumor proliferation and metastasis. Transfection-mediated expression of intracellular nanobodies appears to be a feasible strategy for diagnostic or therapeutic applications for the intracellular antigens. Further, characteristic of nanobodies is still under research.

EFFECT ANALYSIS OF INHIBITORS

Based on the inhibiting mechanism of inhibitors, we conducted a systematic review of the safety and efficacy of inhibitors for the treatment and prevention of cancers in order to provide more information about the inhibitors for both biologists and oncologists. The inhibit efficacy was concluded in Table III and more results could be found in the following references. Some inhibitors have been evaluated in clinical trials, others show antitumor effect in either cell cultured models or animal xenograft models. Investigators concluded that some inhibitors show great potential to be exploited as new antitumor agents, such as YC-1, PX-478, and ENMD-1198. They greatly decrease HIF-1 synthesis and possess good antitumor effect, but have less toxicities and deleterious effects on whole organism. Some inhibitors show potential antitumor effect, but with common adverse events, such as LAQ824 and Flavopiridol (alvocidib). Although, some others are unlikely to have a significant direct cytotoxic effect, it should be studied further for potential chemotherapy enhancing and cytostatic properties, such as Green tea extract (GTE), Echinomycin (NSC-13502), PX-12 and so on. Nanobodies, a kind of new inhibitors, show remarkable promise to be exploited as robust diagnostic and therapeutic agents than chemical inhibitors. Ablynx (Nanobody®, Ghent, Belgium) is the first company that focused on the discovery and development of nanobodies, for a range of serious human diseases including inflammation, hematology, oncology and pulmonary disease. The unique characteristics of nanobodies and continuous innovation have allowed Ablynx, and its partners (e.g., Merck Serono), to develop differentiated drug products, and the drugs will be coming on to the market for clinical application in a few years.

CONCLUSION

Uncontrolled growth of the cancer cells lead to the increasing expenses of oxygen. Consequently, hypoxia is the most common phenomenon in solid tumors. As the central regulator of cells under hypoxia situation, therefore, HIF-1 is an interesting therapeutic target and searching for HIF-1 inhibitors has been an encouraging point of anti-cancer research and received more and more attention. As concluded in Table I, many chemical inhibitors have been identified. But future work is still warranted to identify more HIF-1α inhibitors, study their inhibition mechanism and convert the results

TABLE III. Comparative Analysis of Inhibitors

Agents	Inhibiting effect		Deleterious effect	References
	On HIF-1	On tumor		
YC-1	Inhibit the expression of HIF-1α and the induction of VEGF, aldolase A, and enolase 1 in cancer cells (data was not shown)	YC-1 inhibits MDA-MB-468 breast cancer cells and hypoxic human bladder transitional carcinoma cell line T24 cells	No serious toxicity was observed in nude mice. Did not suppress the cytolytic activity of splenic lymphocytes in vitro or in vivo	Cheng et al. [2012], Li et al. [2012], Yeo et al. [2003]
PX-12/pleurotin	Inhibited the growth of MCF-7 and HT-29 cells, both caused dose-dependent decreases of HIF-1α protein levels.	Anti-tumor effect was well observed in PX-12 and MCF-7 cells xenografts. Clinical trials showed no anti-tumor activity	Overall PX-12 was well tolerated. Grade 3 events were uncommon. Pertinent Grade 1 or 2 toxicities show dose-dependent increase	Ramanathan et al. [2011], Welsh et al. [2003]
AJM290/AW464	Increasing HIF-1α expression but decreasing transcriptional activity, DNA binding and degradation	Antitumor effect was observed in vitro and vivo studies against breast, colon, and renal cell lines or xenografts	Relevant results were not published	Jones and Harris [2006], Jones et al. [2006]
Echinomycin (NSC-13502)	Echinomycin showed antitumor activity against B16 melanoma and P388 leukemia, however, minimal or no antitumor activity was found in phase II clinical trials and it was suspended		Nausea, vomiting, reversible liver enzyme abnormalities, and allergic reactions	Kong [2005], Vlaminck et al. [2007]
Polyamides	Decreased VEGF mRNA and secreted protein levels in a dose-dependent manner in Hela cells	Show promising potency against human cancer cell lines and xenografts possessing human cancer cells	Relevant results were not published	Olenyuk et al. [2004], Shinohara et al. [2010]
Flavopiridol (alvocidib)	It shows promising preclinical and phase I trial results. A phase II trial of combining flavopiridol and cisplatin therapy displayed clinical activity in platin resistant and sensitive ovarian/primary peritoneal cancers, meriting further study. Now in phase III trials		Grade 4 (10%), Grade 3 (65%): Neutropenia (17.5%); nausea (12.5%); vomiting, fatigue, thrombosis, anemia (10% each)	Bible et al. [2012], Bose et al. [2012]
Chetomin	Reduced hypoxia-dependent transcription, carbonic anhydrase 9 (CA9) and VEGF mRNA to 44.4 ± 7.2% and 39.6 ± 16.0%	Chetomin reduces HT 1080 human fibrosarcoma cells in vitro and antitumor effect was well observed in vivo	The function of CTM is not fully understood. Relevant results were not published	Kessler et al. [2010], Kung et al. [2004], Staab et al. [2007]
Bortezomib (Velcade, PS-341)	Bortezomib has been reported to inhibit tumor adaptation to hypoxia by functionally inhibiting HIF-1α	Bortezomib has been approved for the treatment of multiple myeloma and several solid tumors	Asthenia, nausea, diarrhea, appetite decreased, constipation, thrombocytopenia, peripheral neuropathy, vomiting and anemia	Shin et al. [2008]
Amphotericin B	Inhibits the transcription of the EPO gene by inactivating HIF-1α	Approved for severe systemic fungal infections, antitumor effect needs further studies	Nausea, vomiting, loss of appetite, fever, chills, headache; Renal toxicity	Yeo et al. [2006]
LY294002 and Wortmannin	Attenuates HIF-1α protein synthesis in osteosarcoma, renal carcinoma, and prostate cancer cell lines	Well therapeutic effect, especially its derivate of PX-866, now in a phase I clinical trial	Wortmannin has detrimental influence on memory and impair spatial learning abilities. LY294002 causes a substantial acceleration of MEPP frequency	Bowles and Jimeno [2011], Jiang et al. [2001]
Rapamycin, Everolimus and CCI-779	Approved as immunosuppressor. Everolimus significantly inhibited tumor growth, especially combined use with cyclophosphamide. Rapamycin has limited anticancer effect due to its poor solubility and stability in solution. CCI-779 showed activity against a wide range of cancers and is being evaluated in clinical trials		Effective and safe immunosuppressant. Major side effects include headache, nausea, dizziness, nosebleeds and joint pain	Cejka et al. [2008], Hudson et al. [2002], Wan et al. [2006]
Silibinin	It was called as natural hepatoprotective drug and has been approved. It was reported to exhibit anticancer properties through inhibiting HIF-1α accumulation and HIF-1 transcriptional activity		Less toxicities and side effects were observed	Garcia-Maceira and Mateo [2009]
2ME2 and ENMD-1198	ENMD-1198 inhibited tumor growth strongly in metastatic and primary tumor models. A phase I dose-escalation, safety and pharmacokinetic study verified the worth for additional study		2ME2 and its analog are well tolerated with no major toxicities	Mabjeesh et al. [2003], Zhou et al. [2011]
PX-478	Phase I trial was an open-label, dose escalation trial in 41 patients with advanced cancer and designed to examine safety, tolerability, pharmacokinetics, pharmacodynamics and antitumor activity. The results were promising and worth for additional study		Common adverse events are nausea, fatigue, diarrhea and vomiting	Lee and Kim [2011], Welsh et al. [2004]
2-phenethyl isothiocyanate	2-phenethyl isothiocyanate showed chemoprotective effects in a number of animal models of experimental carcinogenesis at various organ sites and against carcinogens of several different types		In a clinical phase I study, no significant or consistent subjective or objective abnormal toxicities were observed	Dinkova-Kostova [2012], Shapiro et al. [2006]
GA and analogs	GA and its derivatives have been reported to possess multiple antitumoral properties, such as on multiple myeloma, breast and prostate cancer. 17-AAG, a GA 'e has been evaluated in Phase II/III clinical trials, and has been found to possess a better stability and to retain a potent anticancer activity even at nanomolar concentrations		GA cannot be evaluated in clinical trials for its hepatotoxicity, poor water solubility and limited oral bioavailability 17-AAG has a lower toxicity	Gorska et al. [2012], Isaacs et al. [2002], Mabjeesh et al. [2002]
Radicicol and its derivants	Radicchio has failed to be effective in animal models. Its derivant of KF58333 showed significant antiproliferative and antitumor properties in KPL-4 human breast cancer xenografts		Relevant results were not published	Hur et al. [2002], Kurebayashi et al. [2001]
Apigenin	Inhibited expression of VEGF mRNA by specifically inhibiting HIF-1α	It has been shown to possess anti-tumor properties in preclinical or clinical trials	It induces resistance against chemotherapy	Fang et al. [2005]

(*Continued*)

TABLE III. (*Continued*)

Agents	Inhibiting effect		Deleterious effect	References
	On HIF-1	On tumor		
Trichostatin A (TSA)	Downregulates hypoxia-response genes by the suppression of HIF-1α activity	Anti-tumor effect was well observed, and now in phase I clinical trial	Relevant results were not published	Yang et al. [2006]
LAQ824	Well anti-tumor effect was observed. It was observed to be well tolerated in a phase I trial. Show great potential as new agent to inhibit HIF-1 synthesis and tumor growth		Less toxicities and side effects	Qian et al. [2006]
SAHA and FK228	Vorinostat (SAHA) and Romidepsin (FK228) were approved for the treatment of cutaneous T cell lymphoma (CTCL). The application for other tumors is still under study		Nausea, vomiting, fatigue, infection and blood disorders, metabolic disturbances, skin reactions	Mie et al. [2003], Shankar et al. [2009]
Wondonin	Decreased hypoxia-induced HIF-1α protein and VEGF expression, inhibited angiogenesis in vitro and in vivo		Relevant results were not published	Jun et al. [2007]
Green tea extract (GTE) and EGCG	Preclinical evidence suggests that GTE may potentiate the anticancer effects of chemotherapy drugs; Two phase I trials assessed the use of GTE in lung cancer patients, however, further investigation is required before GTE can be recommended for this purpose in humans		GTE was well tolerated, with mild side effects, including nausea, restlessness, pain, polyuria and polydipsia at a dose-dependent manner	Fritz et al. [2012], Zhang et al. [2006]
Resveratrol	Resveratrol has been shown to be an effective chemopreventive agent in multiple murine models of human cancers. Much work needs to be done to improve the bioavailability and pharmacologic properties and to better understand its exact mechanisms of action in order to predict its efficacy		Noticeable adverse effects have not been reported within the normal dose-range	Athar et al. [2007], Bishayee et al. [2010]
Small Interfering RNA (siRNA)	siRNA expression vectors can inhibit the expression of HIF-1α and of those genes induced by HIF-1α, thus effectively suppressing the in vitro and in vivo growth of selected hepatobiliary tumors		Relevant results were not published	[Mizuno et al., 2006]
RX-0047	RX-0047 is an effective anti-cancer drug, particularly in a human lung cancer and prostate cancer model, and warrants further investigation that may lead to the design of human Phase I clinical trials		Relevant results were not published	Dikmen et al. [2008]
Anti-HIF-1α and Hetero-bivalent nanobodies (AG1-5)	A new kind of inhibitors of HIF-1α with good targeting and tissue compatibility. Nanobodies have a better antigen binding capacity for its unique crystal structure than conventional antibodies. Nanobodies are screened based on the sensitive screening method and can target HIF-1 specifically		According to the theoretical study, adverse effects to the whole organism should not be observed	Groot et al. [2006, 2008], Vanlandschoot et al. [2011]
Anti-HIF-1α nanobodies (AHPC)				

into clinical application for cancer treatment. However, the main obstacle of the development of chemical inhibitors is the lack of specificity, so it is urgency to identified inhibitors more specifically. The discovering of nanobodies opened a new gate for targeted cancer therapy. The unique structure of nanobodies endowed their good solubility, great antigen binding capacity and stability. It is sure that they will become the potent candidates to biomedical applications and will be the hot point of research now and in the future.

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