

Hypoxia is a commonly occurring event in solid tumours. It triggers specific and non-specific mechanisms enabling the cell to adapt to oxygen deprivation. The most important role in this process is played by hypoxia-inducible factor-1 (HIF-1), which acts as a transcription factor regulating gene expression in hypoxic cells and is overexpressed in most solid tumours. It controls key aspects of cancer biology, which include promoting angiogenesis and erythropoiesis, maintenance of stem cell pools, immortalization, switch to anaerobic metabolism, and promoting cell survival and invasion. HIF-1 itself can be regulated via a number of oxygen-dependent or independent mechanisms, some of which are still poorly understood. It is of great importance to know exactly how HIF-1 is expressed and stabilized, as only this way can efficient HIF-1 inhibitors be provided. This review summarizes mechanisms of HIF-1 control and evaluates anticancer agents based on HIF-1 inhibitors known to date.

**Key words:** HIF-1, hypoxia, HIF inhibitors, tumour angiogenesis, cancer.

# Hypoxia-inducible factor as a transcriptional factor regulating gene expression in cancer cells

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Hypoxia is a state of oxygen deficiency in tissues, which occurs when the oxygen concentration is lower than 5 mm Hg. Hypoxia is caused by the limited capacity of oxygen to diffuse a distance greater than 180  $\mu\text{m}$  [1]. Transcription factor HIF-1 (hypoxia-inducible factor-1) is the main response element for oxygen deprivation. It is expressed in tumour cells while in other tissues its activity is inhibited. HIF-1 overexpression has been observed in most solid tumours – breast, kidney, pancreas, brain, intestines, prostate [2]. Noticeably, there was no expression of this factor in surrounding cells nor in benign tumours [3]. A high level of HIF-1 protein activates many genes responsible for the cell's adaptation to a low level of  $\text{O}_2$ , most of them with an effect on tumour progression, chemoresistance and radioresistance and bad prognosis for the patient [4]. Deactivation of HIF-1 would lead to more effective cancer therapy and better prognosis. Many HIF-1 inhibitors have been discovered. Their mechanism of action varies from regulation of transcription, translation, and posttranslational modifications, to interactions with other transcription factors. The most effective HIF-1 inhibitors are currently being tested in clinical trials.

HIF-1 induces expression of target genes by binding to the DNA sequence 5'-[A/G]CGTG-3', located in the area of HRE (hypoxia responding elements) (Fig. 1). Binding of transcription factor HIF-1 gives rise to a series of subsequent processes leading to cancerogenesis. They include: inhibition of apoptosis, switch to anaerobic metabolism, angiogenesis, cell proliferation, erythropoiesis, and metastasis. To date, over 100 genes have been discovered to be dependent on transcription factor HIF-1.

## Angiogenesis

Cells in the state of hypoxia, such as poorly vascularized tumour cells or stromal cells, under the influence of HIF-1 activate the process of angiogenesis. The main activator of this process is vascular endothelial growth factor (VEGF), which is also one of the most important target genes for HIF-1. Blood flow regulation is not limited only to direct VEGF stimulation. It involves other proteins that are products of genes activated by HIF-1. These proteins regulate, among others, vascular wall tension. These include nitric oxide synthase, haem oxygenase-1, endothelin-1, adrenomedullin and adrenergic receptor  $\alpha_{1b}$  [5, 6].

## Proliferation

HIF-1 activates the expression of growth factors responsible for proliferation and cell survival. The most important of them are insulin-like growth factor 2 (IGF2) and transforming growth factor  $\alpha$  (TGF- $\alpha$ ). By binding to specific receptors IGF1R and EGF1R respectively, they activate a cascade of signals promoting cell survival. Through activation of MAPK and PI3K pathways, they simultaneously lead to increased expression of HIF-1 $\alpha$  [6]. In addition, recent



**Fig 1.** Molecular organization of HIF-1 $\alpha$ .

HIF-1 is a heterodimer consisting of  $\alpha$  (120 kDa) and  $\beta$  (91-94 kDa) subunit. HIF-1 $\alpha$  gene is localized on chromosome 14q21-q24. BHLH (basic-helix-loop-helix) domains enable DNA binding and dimerisation, PAS domains are responsible for dimerisation and target specification. Function of the ODD (oxygen degradation domain) is proteasomal degradation by ubiquitination. Transactivation domains CTAD and NTAD allow for recruitment of transcription cofactors.

studies prove a direct effect of HIF-1 on the expression of anti-apoptotic protein Bcl-xL from the Bcl-2 family [7].

### Glycolysis

Hypoxic cells change their metabolism to anaerobic, acquiring ATP only from the glycolysis pathway. The main activator of this process, the so-called Warburg effect, is HIF-1. Transcription of the pyruvate dehydrogenase kinase complex, (PDK3) inhibits the mitochondrial respiration in favour of glycolysis [8]. The expression of all glycolytic enzymes is determined by transcriptional activity of HIF-1 $\alpha$ . Due to the low energetic yield of glycolysis, an increased amount of glucose must be supplied to the cell. HIF-1 increases the expression of GLUT1 and GLUT3 glucose transporters. To eliminate the acidity caused by anaerobic metabolism, HIF-1 regulates the expression of carbonic anhydrase CA-9.

### Iron metabolism

Iron is an essential substrate for haem formation, and the most common limiting factor in the process of erythropoiesis. HIF-1 induces expression of the iron transporter – transferrin, thereby increasing the amount of iron absorbed by erythroid cells. Transferrin can bind iron only in the form of ions. The process of oxidation of iron to iron ions is mediated by ceruloplasmin, a protein whose expression is dependent on HIF-1 [9].

### Metastasis

The critical moment of carcinogenesis is the acquisition by the cells ability to metastasis. HIF-1 produced in the hypoxic areas of tumours activates the expression of genes regulating the cell potential for metastasis: E cadherin, lysine oxidase (LOX – lysyl oxidase), stromal derived growth factor (SDF-1) and its receptor CXCR4. It has been shown both *in vitro* and *in vivo* that inhibition of LOX, directly activated by HIF-1 expression, is sufficient to prevent hypoxia-induced metastasis [10].

### Inhibition of differentiation

Overexpression of HIF-1 inhibits differentiation of cancer cells. The membrane receptor Notch, which is responsible for the maintenance of undifferentiated cells, is regulated by HIF-1 $\alpha$ . After binding a ligand – such as  $\gamma$ -secretase – the cytoplasmic domain of the Notch protein, containing a nuclear localization signal, is released. Notch activates transcription of target genes necessary to inhibit the differentiation and self-renewal of the cell [11]. Recently, the

role of HIF-2 factor in the process of differentiation of stem cells has been discovered. HIF-2 directly regulates the expression of the transcription factor Oct4, considered one of the four factors responsible for the transformation of fibroblasts into cells with properties of stem cells.

### Drug resistance

HIF-1 plays a significant role in the emergence of drug resistance [6, 12-15], but the exact mechanism of this process is not yet known. Most chemotherapeutic agents kill cancer cells, causing damage to their DNA. Experiments with etoposide (a cytotoxic derivative of podophyllotoxin) demonstrated that hypoxia prevents DNA damage caused by this drug. Induction of HIF-1 reduces the expression of topoisomerase II, an enzyme responsible for degradation of DNA after etoposide. Introduction of siRNA against HIF-1 into cells abolishes the effect of resistance to therapy [16]. HIF-1 also regulates the expression of P-glycoprotein, the protein responsible for multidrug resistance, also known as MDR1 (multiple drug resistance protein). MDR1 acts as an ATP-dependent pump for xenobiotics with broad specificity, inhibiting the accumulation of drugs in the cell.

### Hypoxia inducible factor as a prognostic factor

Expression of HIF-1 $\alpha$  is associated with greater cancer malignancy and poor prognosis [17]. HIF-1 may be expressed in a normoxic state, due to gain-of-function mutations in oncogenes (Ras, Src, Her2, mTOR) or loss-of-function mutations in suppressor genes (VHL, PTEN, p53) [18]. In some tumour cell lines expression of HIF-1 is also stimulated by growth factors produced by the cells. Another possible reason for the increased concentration of HIF-1 $\alpha$  in a cell may be its stabilization by free radicals (ROS – reactive oxygen species) [19].

Change in the expression level of HIF appears to be a good marker for assessing disease progression and aggressiveness of the tumour. Poor prognosis and lack of response to treatment in cells with overexpression of HIF-1 has been confirmed, among others, in head and neck cancers, gliomas, rectal cancer, nasopharynx, pancreas, breast, endometrial and cervical cancer, ovarian cancer, bladder, gastric cancer and osteosarcoma [17].

The main regulator of transcriptional activity of HIF-1 factor is von Hippel-Lindau protein (VHL), which binds to HIF-1 $\alpha$  and directs it to degradation in proteasomes. Inactivation of VHL leads to stabilization of HIF-1 $\alpha$  and expression of its target genes. The effect of mutations in the VHL gene is von Hippel-Lindau disease, which is characterized by familial predisposition to the development of highly vascularized

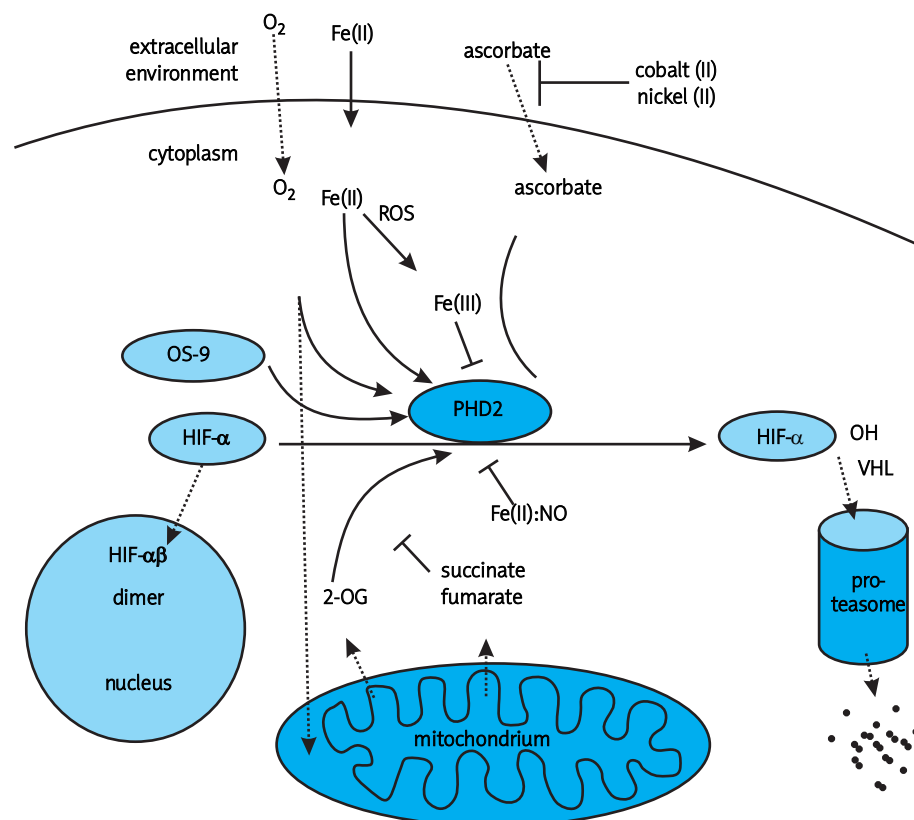
tumours such as retinal and CNS haemangioblastoma, renal cell carcinoma (RCC), pancreatic cancer or pheochromocytoma [20]. The important role of factor HIF-2 in cancer development has been discovered. Tumours derived from renal cancer cell lines overexpressing HIF-2 appeared to grow more dynamically than the ones overexpressing HIF-1 [21]. These conclusions were confirmed on stem cell-derived teratomas, which at the locus for the HIF-1 $\alpha$  gene had the HIF-2 $\alpha$  gene [22]. Overexpression of both HIF-1 $\alpha$  and HIF-2 $\alpha$  is associated with tumour progression. However, there is evidence of the positive effect of their actions, such as activation of proapoptotic genes BNIP3 and NIX.

HIF-1-dependent tumour radioresistance to radiation is caused by the expression of angiogenic cytokines, including VEGF. It was shown that radioactive iodine therapy in combination with antiangiogenic therapy significantly reduces tumour growth through HIF-dependent tumour cell apoptosis [23, 24].

### Regulation of HIF-1 activity in cells

Oxygen-dependant HIF-1 $\alpha$  regulation consists mainly of HIF-1 hydroxylation by PHD (prolyl hydroxylases), which leads to its proteasomal degradation. Under normal conditions, HIF-1 $\alpha$  is also hydroxylated in conservative asparagine residue 803, by the asparagine hydroxylase FIH-1 (inhibiting factor HIF-1). This blocks the binding of cofactors p300/CBP, therefore inhibiting the transcriptional activity of HIF-1 [25]. During hypoxia the lack of oxygen and an increase in anaerobic metabolism cause inhibition of hydroxylation by PHD, thereby increasing the concentration of HIF-1 $\alpha$  (Fig. 2).

Hypoxia is a major, but not the only factor stimulating the expression of HIF-1 $\alpha$ . There are many mechanisms regulating protein levels of HIF-1 $\alpha$  independently of oxygen concentration in the cell. One of them is a pathway involving tumour suppressor protein p53. Under normal conditions, the level of p53 protein in the cell is low, since it binds



**Fig. 2.** Activation pathway of HIF-1 $\alpha$

*In physiological oxygen levels, HIF-1 $\alpha$  is hydroxylated in two proline residues in ODD domain. This process is catalyzed by prolyl hydroxylases, which in the presence of cofactors (ascorbic acid and Fe<sup>2+</sup>) use oxygen and  $\alpha$ -ketoglutarate as substrates. Hydroxylated HIF-1 $\alpha$  is recognized by VHL protein. With other proteins it forms ubiquitin ligase E3 complex. Subsequent polyubiquitination and degradation of HIF-1 $\alpha$  takes place in proteasome 26s. In physiological oxygen levels, ARD1 (arrest defective-1) N-acyltransferase acetylates HIF-1 $\alpha$  in lysine 532, which enables its recognition by VHL. When oxygen levels drops below 5%, hydroxylation of HIF-1 $\alpha$  by PHDs is inhibited, as the concentration of the main substrate of the process – O<sub>2</sub> is insufficient. Production of ROS by oxidative fosforylation increases, it causes iron oxidation, which blocks PHDs. PHD inhibition and HIF-1 $\alpha$  stabilization may also be the effect of Co<sup>2+</sup> and Ni<sup>2+</sup> accumulation and blockade of ascorbate transport. PHDs activity is also inhibited by  $\alpha$ -ketoglutarate analogs. In the hydroxylation process, OS-9 is a helper protein, which binds to PHD2 and PHD3, forming a hydroxylation complex. (Based on: Fong GH, Takeda K. Roles and regulation of prolyl hydroxylase domain proteins. Cell death and differentiation 2008; 15: 635-641).*

to the MDM2 protein (murine double minute 2) undergoing degradation. Under conditions of oxidative stress, an excess of p53 dimerizes and joins a single ODD domain of HIF-1 $\alpha$  factor. This complex is degraded by binding to MDM2. Thus, loss of functional p53 may contribute to the accumulation of HIF-1 $\alpha$ . The p53 protein also affects the transcriptional activity of HIF-1 $\alpha$ . Both HIF-1 $\alpha$  and p53 bind to the p300/CBP complex. In the case of p53 mutation, competition for binding the cofactors does not occur, promoting the transcriptional activity of HIF-1 [4].

HIF-1 protein stability is also regulated by the heat-shock protein (HSP 90) and RACK (receptor of activated protein kinase C) receptor, which compete for binding of HIF-1 $\alpha$ . The HIF-1/RACK1 complex is degraded in the proteasome. HSP90 inhibitors such as 17AAG (17-allylamino-17-demethoxygeldanamycin) cause independent of oxygen and VHL protein decrease in the level of HIF-1 $\alpha$  [26].

The most important regulator of HIF-1 activity is VHL (von Hippel-Lindau) protein. It has been discovered that E2-EPF UCP (E2-endemic pemphigus foliaceus ubiquitin carrier protein) enzyme can specifically ubiquitinate VHL independently of oxygen concentration [27]. UCP is overexpressed in many cancers, which may explain the increased HIF-1 $\alpha$  level observed in tumour areas without hypoxia.

Recent studies confirm participation of a specific E3 ligase, HAF protein (hypoxia-associated factor), in the degradation of HIF-1 $\alpha$  (but not HIF-2 $\alpha$ ) in proteasomes [28]. This degradation is independent of oxygen concentration and the VHL protein. HAF expression was detected in all dividing cells of normal and tumour cell lines. HAF overexpression leads to a significant decrease in the level of HIF-1 $\alpha$  and inhibition of tumour growth *in vivo* [29].

In the normoxic state, translation of HIF-1 $\alpha$  is regulated by cytokines and growth factors. Induction of protein may be dependent on activation of PI3K-Akt pathways, mTOR, and MAPK. It has been proven that the mTOR inhibitors effectively reduce levels of HIF-1 $\alpha$ , but the mechanism of this inhibition is not well understood. It is believed that mTOR regulates the translation of HIF-1 $\alpha$  mRNA through a 5' terminal oligopyrimidine sequence [30]. Other studies suggest direct binding of HIF-1 $\alpha$  with raptor, which is part of the mTORC1 complex [31]. But still it is hard to assume that the main hypoxia response factor is activated by mTOR, which activity is inhibited during hypoxia. An alternative mechanism of HIF-1 $\alpha$  mRNA translation was proposed. It concerns translation dependent on the IRES sequence, but has not been proven yet [32-34].

### Inhibition of HIF-1 in cancer treatment

Knowledge of the detailed mechanisms involving HIF-1 in the process of oncogenesis has led to the use of HIF-1 inhibitors in cancer therapy. HIF-1 inhibitors work by reducing the amount of HIF-1 mRNA, protein, DNA-binding capacity or transcriptional activity. Among all the methods of silencing the expression of HIF-1, the most effective tend to be small molecule inhibitors.

### Small molecule inhibitors

In many tumours the level of HIF-1 $\alpha$  protein synthesis is dependent on the activity of mTOR. Activation of the recep-

tors for tyrosine kinases (HER2, BCR-ABL, EGFR) or AKT and Ras/MAPK pathways leads to increased mTOR activity and increased synthesis of HIF-1 $\alpha$ . Inhibitors of these signalling pathways can induce a therapeutic effect by inhibiting HIF-1 $\alpha$  and other proteins involved in carcinogenesis. Rapamycin (sirolimus, Rapamune), an inhibitor of mTOR, is an immunosuppressant approved by the FDA and used in kidney transplantations. Antiproliferative properties of rapamycin and its use in cancer treatment are being tested [35, 36]. Studies of rapamycin analogue RAD-001 (everolimus) in a transgenic mouse model of prostate cancer showed remission of the disease after administration of an inhibitor of mTOR. Test results from microarray show lower levels of HIF-1 $\alpha$  mRNA and its target genes after mTOR inhibitors [37, 38]. Phase III clinical trials are ongoing. mTOR inhibitor blocking mRNA translation of HIF-1 $\alpha$  is called CCI-779 (temsirolimus). Temsirolimus is an FDA approved drug for kidney cancer. However, after examining the response of patients to the drug it was found that it is not dependent on the HIF-1 $\alpha$  concentration [39, 40]. Clinical trials of Akt, PDK-1 and PI3K inhibitors have been conducted, yet no significant overall survival prolongation was achieved in patients [35].

Many anticancer drugs which reduce HIF-1 $\alpha$  levels in cells have been described. Inhibitors of angiogenesis such as vincristine and 2-methoxyestradiol stimulate microtubular cytoskeleton degradation causing a reduction in the level of HIF-1 $\alpha$ , thereby reducing the level of VEGF, GLUT-1 and endothelin-1. It is assumed that part of the clinical effect of the inhibitors of angiogenesis is due to the inhibition of HIF-1 function [4]. RAS and Src oncogenes are responsible for HIF-1 stabilisation. Inhibitors of farnesyl transferase – an enzyme essential for the activity of Ras proteins – may have a therapeutic effect, increasing the degradation of HIF-1 $\alpha$  [23]. The best known inhibitors of FTS (farnesyl transferase) are Tipifarnib (R115777), Lonafarnib (SCH66336) and BMS214662. Their efficacy in combination with other anticancer agents is currently being tested. It has been proven that inhibition of Raf or MEK genes by sorafenib (BAY43-9006) and RAF-265 – VEGFR and Raf inhibitors – is effective [35].

In 2006, the FDA approved dasatinib (BMS-354825), a double-specific inhibitor of Src and Abl kinases, for the treatment of leukaemias. Studies are being undertaken to enable the use of this drug in the treatment of solid tumours. In the early trials, there are also other Src inhibitors: Bosutinib (SIC 606), AZD-0530, XL-999, and XL-228 [35].

There is a large group of HIF-1 inhibitors whose mechanism of action is not to inhibit the synthesis or promote degradation of HIF-1 $\alpha$ , but to block its transcriptional activity. Examples of such reagents may be echinomycin, polyamides, and DJ12. Echinomycin, a drug with limited specificity, binds preferentially to the sequence 5'-ACGT-3' and 5'-TCGT-3', thereby competing with HIF-1 $\alpha$  for the availability of substrate. Bortezomib (PS-341), a drug used to treat multiple myeloma, is an inhibitor of the proteasome and increases the levels of HIF-1 $\alpha$  by blocking its degradation. Paradoxically, however, it also decreases the transcriptional activity of HIF-1, by blocking the C-terminal transactivation domain of HIF-1 $\alpha$ . The mechanism of this inhibition is not yet fully understood [41].



An inhibiting effect on HIF-1 $\alpha$  expression is shown by a broad spectrum of histone deacetylase (HDAC) inhibitors. Vorinostat (SAHA), used in the treatment of cutaneous T-cell lymphoma, is in advanced clinical trials for the treatment of other cancers. So are the rest of the HDAC inhibitors, such as MS-275, NVP-LAQ824, NVP-LBH589, FR901228.

Screening of the small molecule libraries of the National Cancer Institute led to the discovery of chetomin. Chetomin connects to the CH1 domain of P300 protein and changes its structure, preventing its interaction with HIF-1 $\alpha$ . A significant impact of chetomin on the radiosensitivity of fibrosarcoma cells *in vitro* has also been proven [42].

In summary, there are many small molecule inhibitors that inhibit the action of HIF-1 $\alpha$ . However, most of them work non-specifically and it is not certain that the inhibition of HIF-1 $\alpha$  is responsible for their antitumour effect. Ongoing clinical trials should provide an answer to this question.

Recent reports also suggest that the antitumour activity of natural substances such as celastrol or red ginseng (KRGW) is based on the mechanism of inhibition of HIF-1. Celastrol reduces levels of HIF-1 $\alpha$  mRNA in both the hypoxic and normoxic state, partly by blocking the activity of HSP 90 [43]. Red ginseng, on the other hand, inhibits dimerization of HIF-1 $\alpha$  with ARNT [44].

In addition to small molecular inhibitors, molecular methods for silencing the expression of HIF-1 $\alpha$  are used. These include RNA interference, antisense RNA, ribozymes, splicing variants of mRNA and oligonucleotide traps.

The phenomenon of RNA interference (Nobel prize for Fire and Mello in 2006) is the silencing of the expression of a specific gene by double-stranded RNA. It is a highly specific method of mRNA degradation. Inhibition of HIF-1 $\alpha$  using siRNA is very effective, but the problem of the delivery of siRNA into cells remains. The latest system is based on multifunction EHCO (1-aminoethyl) iminobis [N-(oleicylcysteinyloleic acid) propionamide] transporter. Systemic administration of EHCO/anti-HIF-1 $\alpha$  siRNA nanocomplexes resulted in significantly greater tumour regression compared to intravenous administration of free anti-HIF-1 $\alpha$  siRNA [45].

Studies have also shown the effectiveness of antisense RNA (ASO – antisense oligonucleotides) in cancer therapy. ASO bind to complementary RNA, forming hydrogen bonds – a physical barrier that prevents translation. Administration of the plasmid carrying HIF-1 $\alpha$  antisense RNA into the tumour leads to a decrease in VEGF expression and tumour vascularity reduction [46]. The efficiency of this process, however, is far lower than the efficiency of RNA interference.

Oligonucleotide decoys are another method of regulating the activity of HIF-1 $\alpha$ . These short, double-stranded DNA fragments contain binding elements for the target genes of HIF-1 $\alpha$ . Application of HIFDs (hypoxia-inducible factor decoys) in experimental models yielded good results, including in combination with Avastin [47]. Hammerhead ribozymes directed against HIF-1 $\alpha$  mRNA have also been tested. Ribozymes are molecules of ribonucleic acid which have catalytic properties, enabling them to cut specific target RNA. Angiogenesis and tumour growth after the application

of ribozymes were inhibited [48]. These results indicate the potential of ribozymes for use in targeted therapy.

Alternative splicing forms of HIF-1 $\alpha$  mRNA (e.g., not containing transactivation domains) can act as inhibitors of HIF-1 $\alpha$  through competitive dimerization with ARNT and binding to DNA of target genes. One of these forms is induced by the zinc form of HIF-1 $\alpha$  Z without exon 12. Direct connection to exons 11 and 13 generates a stop codon. The resulting protein is composed of 557 amino acids and has no transactivation domains, NLS signal and part of the ODD domain. A similar variant occurs naturally in various human cell lines and was named HIF-1 $\alpha$ 516. It is a form with deletion of two exons – 11 and 12. The resulting protein of 516 amino acids also does not have transactivation domains [23]. Another inhibitor, an IPAS protein (transcription variant of HIF-3 $\alpha$ ), contains bHLH and PAS domains responsible for dimerization and binding to DNA. It functions as a negative regulator of HIF-1, but the mechanism of this inhibition needs to be clarified [49].

Imperfection of *in vivo* gene transfer methods is an important limitation of anti-HIF-1 gene therapy. However, the high specificity of genetic methods with negligible toxicity of therapy suggests the possibility of wider use of genetic engineering in the inhibition of HIF-1 in future.

An accurate analysis of signal transduction pathways regulating the expression of HIF-1 is a prerequisite for obtaining an effective HIF-1 inhibitor. It needs to be remembered though, that effective cancer therapy will most likely be a combination therapy, and HIF-1 inhibitors may only be a part of it.

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