Heme Oxygenase-1 in Tumor Biology and Therapy

Halina Was, Jozef Dulak and Alicja Jozkowicz[*]

Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

Abstract: Heme oxygenase-1 (HO-1) degrades heme to carbon monoxide (CO), biliverdin, and ferrous iron. As HO-1 expression is highly increased by stressful conditions, the major role of the enzyme is the protection against oxidative injury. Additionally, it regulates cell proliferation, modulates inflammatory response and facilitates angiogenesis. Beneficial activities of HO-1 have been recognized in many pathological states e.g. atherosclerosis, diabetes, ischemia/reperfusion injury or organ transplantation. Interestingly HO-1 expression is very often boosted in tumor tissues and could be further elevated in response to radio-, chemo-, or photodynamic therapy. A growing body of evidence suggests that HO-1 may play a role in tumor induction and can potently improve the growth and spread of tumors. This review discusses the implications of HO-1 properties for tumor proliferation and cell death, differentiation, angiogenesis and metastasis, and tumor-related inflammation. Finally, it suggests that pharmacological agents that regulate HO activity or HO-1 gene silencing may become powerful tools for preventing the onset or progression of various cancers and sensitize them to anticancer therapies.

Keywords: Heme oxygenase-1, carcinogenesis, oxidative stress, cytoprotection, angiogenesis, metastasis.

CARCINOGENESIS

Transition from normal to pre-cancer and cancer cells is a result of multi-step accumulation of genetic and epigenetic modifications. In many types of cancers the aneuploidy is detected and pre-malignant lesions with aneuploidy can transform into malignancy more frequently than that with normal DNA content [1]. Growing body of evidence suggests that aberration in the number of chromosomes is a cause rather than a consequence of malignant transformation [2]. Also the loss of heterozygosity, defined as a loss of genomic material in one of a pair of chromosomes in regions that include tumor suppressor genes, is an early predictor of transformation of pre-cancerous lesions [3]. Finally, the telomerase activity, necessary for maintenance or extension of telomeres, is generally not found in normal tissues, but occurs in low levels in pre-cancerous lesions and in cancers [4].

Deregulations or mutations of genes responsible for cellular growth and differentiation, such as growth factors, receptor or cytoplasmatic tyrosine kinases, serine/threonine kinases, regulatory GTPases, and transcription factors, play a chief role in tumor development. Oncogenes encoded by mutated genes do not possess important regulatory elements and their production and activation is uncontrolled [5]. Cancer-related genes include also tumor suppressors. Their protein products either diminish the cell cycle or favor apoptosis. It has been shown that tumor suppressor genes are important targets in sensing and responding to DNA damage in the multi-step process of carcinogenesis [6, 7].

One of the critical processes correlated with malignancy is angiogenesis. It is a crucial event not only for growth of primary tumors but also for metastasis. Tumor-associated angiogenic factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), are generated both by tumor cells and by infiltrating leukocytes [8]. Recently, a role of tumor vasculogenesis was also indicated [9].

An inflammatory microenvironment is an essential component of all tumors [10]. Inflammation influences different stages of tumorigenesis, including initiation, promotion, malignant conversion, invasion, and metastasis. It also affects immune surveillance and responses to therapy [11]. Bacterial and viral infections [12], tobacco smoke [13], obesity [14, 15], patient age [16], and cell senescence [17] are postulated to be the tumor promoters that act through inflammatory mechanisms.

Final important aspect of tumorigenesis is an oxidative stress that can modulate all steps of the process. It is induced by reactive oxygen species (ROS), including not only the oxygen radicals (superoxide anion radical, hydroxyl radicals, etc.) but also some non-radical derivatives (hydrogen peroxide, singlet oxygen, alkyl peroxide etc.) [18]. Strong oxidative stress leads to cytotoxicity, inhibits cell proliferation and results in apoptotic/necrotic cell death, whereas low or intermediate amounts of ROS cause DNA damage and mutations, and induce inflammatory reaction, leading to augmented cell proliferation and increasing risk of carcinogenesis [19]. ROS levels are precisely regulated by endogenous defense systems, including intracellular superoxide dismutase (SOD), catalase, glutathione peroxidase, and heme oxygenase-1 (HO-1) [20].

EXPRESSION AND ACTIVITY OF HEME OXYGENASES

Heme oxygenases, the rate-limiting enzymes in heme catabolism, catalyze the stereospecific degradation of heme to biliverdin, with the concurrent release of ferrouse iron

*Address correspondence to this author at the Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland; Tel: +48-12-664- 6411; Fax: +48-12-664-6918; E-mail: alicja.jozkowicz@uj.edu.pl

ions and carbon monoxide (CO) [21]. In addition to their role in regulating the cellular levels of heme, HO is responsible for the recycling of iron from senescent red blood cells. Released iron induces the expression of the iron-sequestering ferritin and activates Fe-ATPase, an iron transporter, which reduce intracellular Fe^{2+} content [22]. The second end-product of HO activity, CO, is exhaled from the organisms through the lung [23]. Finally, biliverdin is converted to bilirubin by the cytosolic enzyme biliverdin reductase (BvR). Bilirubin is subsequently oxidized by cytochrome P450 enzymes or glucoronidated by UDP-glucuronyl transferase and then excreted as bilirubin glucoronides into the bile [24, 25] (Fig. **1**).
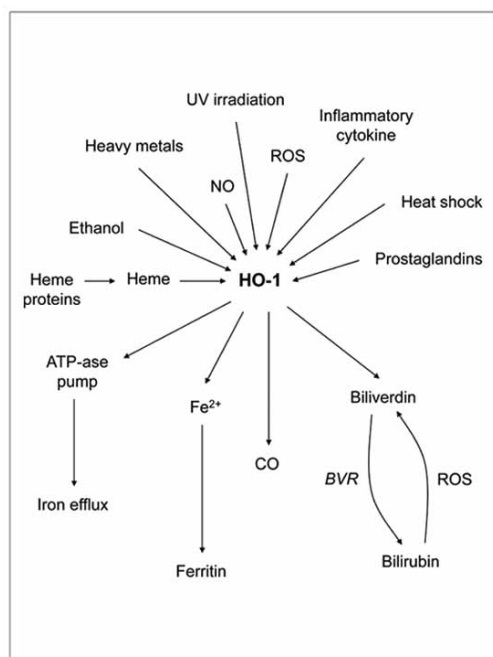

Fig. (1). Schematic demonstration of HO-1 pathway.

Heme oxygenase was first purified to homogeneity from rat liver and then from porcine and bovine spleen [26-28]. Subsequently it was detected in human tissues [29]. There are two isoforms of HO, which are encoded by two distinct genes, but share approximately 40% of amino acid sequence homology [30, 31]. The originally isolated enzyme, with molecular mass of 32 kDa, is designated as HO-1, whereas the second protein, with molecular mass of 36 kDa, is designated as HO-2 [32, 33]. HO-1 is the product of one transcript, but HO-2 is encoded by two transcripts of the single gene which differ in the use of the polyadenylation signal [34].

HO-2 is a constitutively expressed protein, detected at high levels in the brain and, at lower levels, also in testes, endothelium, distal nephron segments, liver, and gut myenteric plexus [32, 33]. In contrast, HO-1 can be strongly induced in many tissues in response to cellular stress caused by a wide spectrum of stimuli, including heme [35], heavy metals [35], UV irradiation, ROS [36], nitric oxide (NO) [37], inflammatory cytokines [38], heat shock [39], ethanol [40], and prostaglandins [41] (Fig. **1**). It can also be activated by hypoxia, but this effect is tissue- and species-dependent [42, 43]. On the other hand, HO-1 seems to be constitutively expressed in renal inner medullary cells [44], Kupffer cells

in the liver [45], Purkinje cells in the cerebellum [46], and $CD4^+/CD25^+$ regulatory T lymphocytes [47]. Enzymatic activity of HO-1 reduces oxidative stress, diminishes inflammatory response, and lowers the rate of apoptosis. Namely, it enables the removal of heme, a potentially toxic prooxidant molecule, allows decreasing a lipid-soluble transmissible form of iron, and leads to the generation of biologically active products.

Heme is an essential iron complex responsible for oxygen and electron transport. It can be released from the heme proteins and, together with the released iron, may catalyze free radical reactions, being harmful to the cells. The major source of heme are erythrocytes, therefore the blood vessels are at the greatest risk of exposure [48]. The hydrophobicity of heme analogues (ferriporphyrins) is decisive for entry into cells and necessary for oxidative injury. It has been shown that ferrohemoglobin can readily be oxidized to heme-releasing ferrihemoglobin in the presence of inflammatory cell–derived oxidants [49-51]. Additionally, heme originating from hemoglobin can interact with NO to enhance heme uptake [52]. Importantly, heme induces neutrophil activation [53], augments endothelial cell adhesion molecule expression and subsequently triggers robust inflammatory reactions [54, 55]. The uptake of heme and resulting augmentation of cellular oxidant vulnerability are both blocked by the heme-binding proteins, such as hemopexin [56, 57], albumin, and haptoglobin [58].

Degradation of heme by HO is the major source of CO, previously regarded as a toxic air pollutant, but currently recognized as an important mediator which, similarly to NO, plays a role in neurotransmission and muscle relaxation. It appears that HO-1-derived CO and bilirubin exert a vasorelaxant effect not only *via* a cGMP-dependent pathway but also *via* a cGMP-independent activation of voltage-gated K^+ channels [59] or indirectly through an increase in adiponectin levels [60]. Due to induction of soluble guanylyl cyclase (sGC), CO also inhibits platelet aggregation, decreases leukocyte adhesion, and reduces endothelial cell apoptosis. Finally, anti-inflammatory properties of CO are related to reduced production of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), or macrophage inflammatory protein-1β (MIP-1β), and concomitant upregulation of anti-inflammatory IL-10 [61].

Ferrous iron ions, the second product of heme degradation, can be dangerous, taking a part in the Fenton reaction to generate toxic hydroxyl radicals, which can oxidize lipids and damage the DNA and proteins. However, iron can be sequestered by ferritin or removed by ATP-dependent pumps, preventing oxidative stress [62]. At the end, both biliverdin and bilirubin, which have been long considered as toxic end-products of heme metabolism, turned out to be the potent antioxidants and inhibitors of complement cascade. Thus, unconjugated bilirubin is capable of scavenging the singlet oxygen and can act as a reducing agent for certain peroxidases, including horseradish peroxidase and prostaglandin H synthase [24].

EXPRESSION OF HO-1 IN TUMORS

Expression of HO-1 is usually higher in cancer cells than in surrounding healthy tissues, as shown for lymphosarcoma

[63], prostate cancers [64-66], brain tumors [67], adenocarcinoma [68], hepatoma [69, 70], squamous carcinoma [71, 72], glioblastoma [73], melanoma [74], Kaposi sarcoma [75] or pancreatic carcinoma [76]. It can be induced by some oncogenes, such as viral G protein-coupled receptor (vGPCR) encoded by HHV-8, a Kaposi sarcoma-associated herpes virus [77]. Also the BCR/ABL fusion kinase, typical for chronic myeloid leukemia, was demonstrated to upregulate HO-1 [78]. Concomitantly, chronic arsenic-induced malignant transformation of rat liver epithelial cells increases the levels of oncogenes, namely α-fetoprotein (AFP), Wilm's tumor protein-1 (WT-1), c-jun, c-myc, and H-ras, which is accompanied by elevation of HO-1 [79]. Of importance, co-expression of c-Myc and TGFα in the mouse liver, that accelerates hepatocarcinogenesis, was related to upregulation of HO-1. The effect was abolished by supplementation with vitamin E, suggesting the role of ROS [80] (Table **1**). Noteworthy, tumors showing strong neovascularization and massive hemorrhages contain significant amounts of heme-releasing oxidized hemoglobin [81]. Heme can directly induce HO-1 expression, what is a protective mechanism against brain [82], intestine [83] or hepatic oxidative injury [84]. Finally, expression of HO-1 in cancer cells can be further elevated in response to chemotherapy, irradiation [76, 85], or photodynamic therapy [86-89].

Table 1. Elevated Expression of HO-1 in Tumors

Type of Cancer	References
Lymposarcoma	[63]
Prostate cancer	[64-66]
Brain tumor	[67]
Adenocarcinoma	[68]
Hepatoma	[69, 70]
Squamous carcinoma	[71, 72]
Glioblastoma	[73]
Melanoma	[74]
Kaposi sarcoma	[75]
Pancreatic cancer	[76]
Chronic myeloid leukemia	[78]

The exact location of HO-1 in transformed tissue may depend on type of tumor. As found in human melanomas [74] or in rat and human gliomas [73] HO-1 was almost exclusively expressed in macrophages, which accumulated in perinecrotic areas [73]. Interestingly, macrophages of non-small cell lung cancer exhibited decreased level of HO-1 in comparison to tumor-free lung macrophages [90]. On the other hand, in rat hepatoma HO-1 was found only in tumor cells [69]. Furthermore, expression of HO-1 in hormone-refractory prostate cancer (HRPCA) was much higher than in localized prostate cancer (PCA) or benign prostate tissues, while there were no such differences in stromal cells surrounding the tumors [66]. Finally, human pancreatic carcinoma displayed marked HO-1 immunoreactivity both in cancer cells and in immunocytes [76].

Recently, a nuclear localization of HO-1 has been shown in human prostate cancer [65, 91], especially in cells treated with hemin [65]. For the first time, it was observed in murine fibroblast and human hepatoma cell lines, where HO-1 translocated to the nucleus leading to activation of oxidant-responsive transcription factors, including activator protein-1 (AP-1). Thus, it was postulated that the nuclear form of HO-1, although enzymatically inactive, may upregulate genes that promote cytoprotection against oxidative stress [92]. Additionally, the nuclear localization of HO-1 was demonstrated in astroglia and implicated in brain development or neurodegenerative diseases [93]. It was also found in rat fetal lung cells exposed to hyperoxia, where it could act as a chaperone or nuclear messenger [94], and in brown adipocytes, where it was suggested as a transcription factor playing a role in adipogenesis [95].

ROLE OF HO-1 IN TUMOR CELL PROLIFERATION

HO-1 is highly upregulated in fast proliferating cells such as epithelium within the wounded skin or psoriatic lesions [96] and cancer cells in the growing tumors [64]. It also seems to affect the cell cycle progression, but in a cell-type specific manner. Thus, HO-1 stimulated the proliferation of keratinocytes [97], vascular endothelium [77, 98], or regulatory CD4$^+$CD25$^+$ T lymphocytes [99, 100], and these effects were reversed by its pharmacological inhibitor (tin protoporphyrin-IX, SnPPIX) or by siRNA [97]. In contrast, HO-1 attenuated divisions of fibroblasts [101], smooth muscle cells [102-104], epithelial cells [105], effector T lymphocytes [106], and astroglia [107]. Again, this effect could be reversed by HO-1 inhibition, confirming the HO-1 specificity [102].

Similarly in tumors, the influence of HO-1 on proliferation depends much on the cell type. Its direct pro-mitogenic action has been shown in pancreatic cancer, where siRNA-mediated downregulation of HO-1 was coupled with a statistically significant inhibition of growth [76], and in murine or human melanoma, where overexpression of HO-1 led to increased cell proliferation [108]. Also, enhancement in divisions of transformed endothelial cells infected with HHV-8 resulted from induction of HO-1, as it was abolished by HO-1 inhibitor (chromium mesoporphyrin-IX, CrMPIX) [75]. Pharmacological or genetic inhibition of HO-1 reduced also the proliferation of prostate carcinoma [66], hepatocellular carcinoma [70], and neoplastic mast cells [109]. Finally, knockdown of HO-1 caused marked growth inhibition in urothelial cancer cell line [88].

In turn, the antiproliferative actions of HO-1 have been demonstrated for lung adenocarcinoma, breast cancer and prostate cancer cells. Namely, HO-1 inhibition with SnPPIX reversed the growth arrest observed in A549 lung adenocarcinoma overexpressing HO-1 [110]. Similarly, application of SnPPIX resulted in a small but significant increase in proliferation of rat and human breast cancer cell lines, whereas induction of HO-1 with cobalt protoporphyrin-IX (CoPPIX) or heme, as well as transduction of cells with lentiviral vectors coding for HO-1 led to growth reduction [111]. Finally, a significant decrease in cell proliferation was observed in hemin-treated or HO-1 overexpressing prostate carcinoma [91]. It must be stressed, however, that employ-

ment of complementary methods, such as siRNA application that allow for specific HO-1 inhibition, is necessary to get the conclusive results, because pharmacological modulators of HO-1 activity may exert many HO-1 independent effects [112].

Of importance, proproliferative effects of HO-1 have been confirmed *in vivo*. Most experiments supported the permissive role of HO-1 in tumor growth, although in some cases, for instance in some prostate cancers, HO-1 overexpression might reduce the mitotic index [91]. Usually, pharmacological or genetic induction of HO-1 facilitated tumor progression, as manifested by bigger volumes of nodules or by more numerous cancer cells. Such a correlation has been demonstrated in angioma [77], and melanoma [108]. In accordance, reduced HO-1 expression resulted in decreased growth of hepatoma, sarcoma [69], pancreatic cancer [76, 113], angioma [77], lung cancer [114], hepatocellular carcinoma [70], and some prostate cancer lines [66].

Exact mechanisms responsible for modulation of cell cycle by HO-1 still require explanation. Nevertheless, microarray analysis of murine melanoma transcriptome showed that augmented proliferation of HO-1 overexpressing cells can be associated with lower levels of p21, the major negative regulator of cell cycle, and with downregulation of Mdm2, the inhibitor of p53 [108]. Apart from other known functions, the p53 plays a role in regulation of oxidative stress, through activation of numerous genes involved in production of ROS. Concurrently, ROS can further activate p53, which may lead to upregulation of cytoprotective and DNA repairing genes [115]. One of such antioxidative responders is HO-1, which contains the p53 responsive element in the promoter sequence [115].

Low levels of ROS can facilitate the reaction to mitogens [116], and HO-1 has been identified as a mediator of ROS-augmented proliferation in the breast cancer cells [117]. Moreover, silencing the HO-1 gene in HRPCA cells, using siRNA or pharmacological inhibitor OB-24, decreased oxidative stress, reduced MAPK (ERK1/2 and p38) signaling, and led to inhibition of prostate cancer proliferation both *in vitro* and *in vivo* [66]. Interestingly, HO-1 was stimulated in a p53-dependent manner in irradiated mice, and irradiation dose required to its induction was higher than that for upregulation of p21. Furthermore, HO-1 response occurred later than the stimulation of p21. It may suggest the existence of a negative feedback loop between the activation of p53 by ROS and the p53-dependent expression of HO-1: increased HO-1 would reduce intracellular oxidative stress leading to diminution of p53 activity and consequently to a decrease in p53 target genes, including p21 [115].

The cyclin-dependent kinase (Cdk) inhibitor p21 is often downregulated in tumors, and its binding to Cdk2 blocks the progression of cell cycle [118]. Importance of p21 attenuation has been already revealed in clinical studies, e.g. in cutaneous malignant melanoma [119]. HO-1 activation attenuates p21 expression in different cell types, including endothelial cells, melanoma, and colon carcinoma [108, 120, 121]. However, there are some studies showing that overexpression of HO-1 results in a significant increase in the levels of p21 in tumors [122, 123]. Important aspect in tumor biology is also a subcellular localization of p21. Recently, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), the

most potent carcinogen of cigarette smoke, has been shown to up-regulate the HO-1 expression and simultaneously induce proliferation of lung cancer cells. Noteworthy, such a treatment promoted the nuclear localization of p21. In accordance, troglitazone (activator of peroxisome proliferator-activated receptor-γ, PPARγ) that is known to arrest the growth of lung cancer, increased the cytosolic fraction of p21 [120]. Nuclear-localization of p21 was reported to correlate with progression of tumors and worsen survival of patients [121]. Importantly, p21 nuclear translocation in cells treated with NKK was completely blocked by HO-1 inhibitor, zinc protoporphyrin-IX (ZnPPIX) [120].

Finally, it was observed that ZnPPIX may reduce activity of transcriptional factors, Sp1 *(Stimulatory protein-1)* and Egr1 (*Early growth response factor-1*), and thereby inhibit the transcription from the cyclin-D1 promoter, suggesting that HO-1 may upregulate the cyclin-D1 generation [124]. This supposition has been confirmed recently in fibroblasts isolated from HO-1 deficient mice, where cyclin-D1 expression was significantly lower than in their wild-type counterparts [Was *et al.*, paper under revision]. Thus, attenuation of D1-dependent activities can be also one of mechanisms underlying the HO-1-dependent regulation of cell proliferation.

There is no single mediator of the proproliferative action of HO-1. In endothelial cells the most important seems the generation of CO and downregulation of p21 [98, 125]. Confusingly, in vascular smooth muscle cells (VSMC) p21 was identified as a protein mediating an HO-1-dependent inhibition of proliferation, and antiproliferative potential of HO-1 was significantly reduced in VSMC obtained from p21 null mice [102]. Additionally, treatment of wild-type VSMC with hemin downregulated the expression of cyclin-D1 and upregulated that of p21, the effects opposite to those observed in endothelium and many cancer cells [126]. Similarly as in VSMC, the positive correlation of HO-1 and p21 was demonstrated in epithelial cells [44], papillary thyroid carcinoma [122], gastric cancer cell line [123], pancreatic [127], and hepatic stellate cells [128]. Again, the postulated effector molecule in antiproliferative action of HO-1 was CO which stimulated p38-MAPK through activetion of sGC and elevation of cGMP. Constitutive activity or overexpression of p38-MAPK can result in permanent cell-cycle arrest and premature cell senescence [129].

One can also hypothesize that CO may influence cell proliferation through caveolin-1 (cav-1) [130], the main structural component of caveolae [131]. Increased expression of cav-1 is characteristic for aged animals and for fully differentiated or senescent cells [132], while its generation is reduced in tumors [133]. In VSMC and fibroblasts the CO activates p38β-MAPK isoform, which leads to upregulation of cav-1 and inhibition of proliferation [130]. Interestingly, HO-1 has been shown to localize in caveolae, and cav-1 seemed to negatively regulate the enzymatic activity of HO-1 in pulmonary endothelium [134].

The second effector product in antiproliferative activity of HO-1 can be biliverdin. This compound is very effectively converted to bilirubin, which in turn can inhibit expression of cyclin A, D1, and E, as well as cdk-2, and block hyperphosphorylation of retinoblastoma protein (Rb) [103]. Additionally, bilirubin inhibits phosphorylation of p38-MAPK

[129] and of ERK1/2, the kinases involved in regulation of cell proliferation [135]. It should be kept in mind, however, that p38-MAPK may be both a permissive [100] and inhibitory regulator of cell cycle progression, depending on the cell contex and/or p38-MAPK isoform active [136].

Finally, reduction in cellular heme content and resulting iron shortage in cells with a very high levels of HO-1 may also lead to inhibition of tumor growth [137]. Iron is essential for proliferation, especially in rapidly dividing cancer cells [138]. Iron chelators such as desferroxamine (DFO) already showed anti-tumoral potential in many tumors *in vitro* and in limited number of clinical trials [139]. Thus, DFO inhibited growth of human myeloid leukemia and neuroblastoma cell lines [140], and displayed anitumor activity in neuroblastoma patients [141]. Finally, DFO significantly improved effectiveness of gemcitabine treatment in mice bearing the pancreatic cancer [142].

Hitherto, data on role of HO-1 end-products in proliferation of cancer cells are very limited and still confusing. Thus, CO exposure led to a tendency towards increased division of pancreatic cancer cells [142], whereas it blocked proliferation of prostate cancer cell lines, and the latter effect was dependent on inhibition of Rb phosphorylation [143]. In turn, application of both biliverdin and ferrous histidinate resulted in increased pancreatic cancer cell proliferation [142]. The well-documented, but conflicting reports regarding effects of HO-1 on cell cycle suggest that the final output does not depend on HO-1 alone, but is rather determined by the reciprocal balance of several players, including HO-1, p21, p53, ferritin, caveolin-1, and possibly other, still not recognized factors (Fig. **2**).
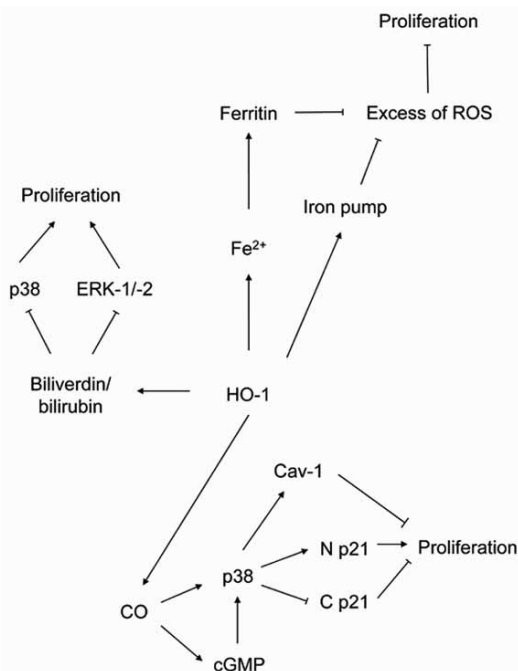

Fig. (2). Schematic demonstration of the role of HO-1 in regulation of cell proliferation in tumors. N - nuclear, C - cytoplasmatic.

ROLE OF HO-1 IN TUMOR DIFFERENTIATION

The role of HO-1 in cell differentiation is not well recognized. Nevertheless, there are some data showing the

influence of HO-1 on maturation of osteoclasts, osteoblasts, dendritic cells, and adipocytes. It was demonstrated that activation of HO-1 prevents differentiation of osteoclasts [144], and inhibits the maturation of primary osteoblasts, as illustrated by attenuated mineralized bone nodule formation, reduced alkaline phosphatase activity and decreased expression of several differentiation markers such as alkaline phosphatase, osteocalcin, and RUNX2 [145]. Similarly, HO-1/ferritin system prevented inorganic phosphate-mediated calcification and osteoblastic differentiation of human smooth muscle cells [146], and depressed the maturation of antigen-presenting cells into monocyte-derived dendritic cells [147]. On the other hand, upregulation of HO-1 shifted the balance of mesenchymal stem cells (MSC) differentiation in favor of osteoblast lineage, whereas HO-1 inhibition drove them towards adipogenesis [148].

The role of HO-1 in cancer cell differentiation is even less established. However, changes in HO-1 expression were shown in erythroleukemia, in which the induction of erythroid maturation by dimethyl sulfoxide (DMSO) was coupled with a rapid decline in mRNAs for HO-1, heat-shock protein-70 (Hsp-70), and nonspecific δ-aminolevulinate synthase (ALAS) [137]. Changes in HO-1 expression were also found in the differentiating myeloid leukemia cell line, which can develop bidirectionally i.e., to erythrocytes after treatment with hemin and to monocytes in response to 12-O-tetradecanoylphorbol 13-acetate (TPA). Interestingly, inhibition of HO-1 activity by SnPPIX suppressed only the TPA-induced maturation to monocytes. Therefore, HO-1 could be suggested as a directional switch and permissive enzyme in monocytic differentiation program of myeloid leukemia [149].

Effects of HO-1 on cell differentiation seem to be associated with redox signaling. Recent studies suggest that production of ROS can be essential for neuronal maturation. Elevation of ROS in haematopoietic progenitors was also found to trigger differentiation into mature blood cell types, through a signaling pathway that involves JNK and FoxO activation [150]. Similarly, hemin-induced ROS may activate AKT and ERK signaling pathways, thereby promoting a significant increase in neovessel formation and expression of endothelial markers in endothelial progenitor cells (EPC) [151]. It can also potentate pro-differentiating potential of BML-210, a histone deacetylase inhibitor, in human leukemia cell lines [152]. Increased activity of HO-1, through degradation of hemin and decrease in ROS generation, might attenuate these effects. It appears that HO-1 might protect cells from oxidative stress-related injury during differentiation but, if expressed at a high level, the enzyme by effective removal of ROS might inhibit cell differentiation. Nevertheless, such a postulate is still mostly speculative and needs to be supported by strong experimental data (Fig. **3**).

ROLE OF HO-1 IN TUMOR CYTOPROTECTION AND APOPTOSIS

HO-1 is activated by many stress-inducing factors and this response may afford protection from oxidative damage and reduce the rate of apoptosis in different cells [153-155]. The most convincing illustration of cytoprotective properties
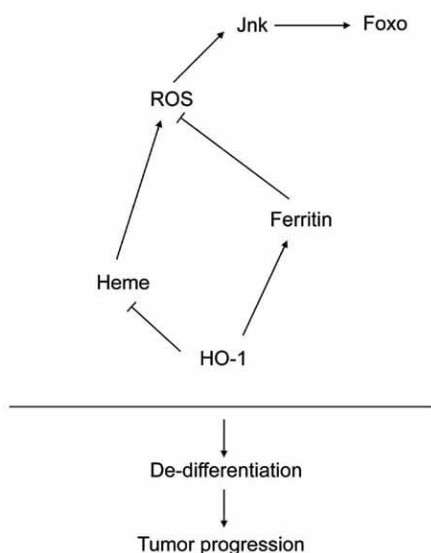
Fig. (3). Schematic demonstration of the role of HO-1 in regulation of cell differentiation in tumors.

of HO-1 come from the studies on HO-1 knockout mice, where HO-1 deficiency leads to sever oxidative stress, increased lipid peroxidation, enhanced oxidative damage in cardiovascular system, and progressive chronic inflammation in the kidney and liver. Moreover, fibroblasts isolated from HO-1$^{-/-}$ individuals demonstrate augmented production of ROS and reduced viability when exposed to oxidants such as hemin or H_2O_2 [156]. Of importance, cytoprotective effects of HO-1 have been confirmed in various diseases, including atherosclerosis, ischemia-reperfusion and acute renal injury, toxic nephropathy, cisplatin nephrotoxicity or endotoxic shock [157].

Cytoprotective and antiapoptotic properties of HO-1 were also demonstrated in cancer cells. Accordingly, pharmacological or genetic activation of HO-1 significantly improved survival of hepatoma [69, 70], thyroid carcinoma [122], chronic myelogenous leukemia [78], gastric carcinoma [123], colon carcinoma [158], melanoma [108], lung carcinoma [159, 160], and glioma [161], whereas HO-1 inhibition reduced viability of colon carcinoma [158], acute myeloid leukemia [162], and hormone-refractory prostate cancer [66].

Of importance, expression of HO-1 is additionally elevated in response to anticancer treatments [76, 85-87, 89], thereby augmenting the resistance of cancer cells [78, 86, 163]. On the other hand, the contribution of HO-1 in tumor cytoprotection allows for increasing the sensitivity of cancer cells to therapeutic procedures by inhibition of HO-1 expression and/or activity. The feasibility of such an approach has been revealed in cultured tumor cells of pancreatic cancer subjected to radiotherapy and chemotherapy [76], in colon carcinoma [164], lung carcinoma [160], and chronic myelogenous leukemia [78, 165] treated with chemotherapy, or in T-cell leukemia [166], melanoma [167], and urothelial carcinoma [88] exposed to photodynamic therapy. Results of *in vitro* experiments have been confirmed in animal models. Particularly, administration of ZnPPIX or ZnPPIX-poly-

ethylene glycol (ZnPPIX-PEG) led to enhanced apoptosis in rat hepatoma and sarcoma or in murine lung tumors [168], while HO-1 siRNA treatment significantly increased the rate of apoptosis in murine hepatocarcinomas [70]. Finally, it was reported that most nasopharyngeal carcinoma patients with undetectable expression of HO-1 were responsive to radiotherapy, whereas almost 60% of those with tumors positive for HO-1 showed high resistance to the treatment [169]. These findings may indicate the HO-1 as a helpful marker in classifying the patients to radiotherapy, and may suggest the inhibition of HO-1 as a new sensitizing strategy for nasopharyngeal carcinoma.

Mechanisms underlying the cytoprotective effects of HO-1 in tumor cells are still not fully understood. One possible pathway is removal of free heme, a prooxidant and important modulator of signal transduction [170]. Heme can be harmful to the cells, but in the same time it directly regulates the activity of Nrf2 and Bach1 transcription factors [171, 172], inducing the expression of not only HO-1 but the whole set of cytoprotective and detoxifying genes [173, 174]. Because of such a complex response it is not trivial to assess the real contribution of HO-1 in cell protection. For example, apoptosis induced by imatinib (ABL tyrosine kinase inhibitor) in leukemia cell lines was suppressed following the treatment with hemin or with δ-aminolevulinic acid (δ-ALA), the heme precursor [134]. Furthermore, hemin selectively counteracted the imatinib-induced repression of antiapoptotic Bcl-2a and Bcl-2b genes [175], and decreased the sensitivity of cells to anthracyclins [163]. The cytoprotective effects were reduced by Nrf2 siRNA, indicating the significance of this transcription factor, but without explanation the role of HO-1 [163]. Nrf2 was also activated in leukemic cells in response to TNF, what augmented the cell resistance to TNF-induced apoptosis. Here, the application of HO-1 siRNA, which restored cell sensitivity, evidenced the role of HO-1 in Nrf2-mediated cytoprotection [162]. Nrf2 signaling pathway was also responsible for induction of HO-1 and protection against NO-induced damage in colon carcinoma cells [174]. Similarly, Nrf2 mediated the HO-1 upregulation and resulting cytoprotection in monocytes treated with epigallocatechin 3-gallate (EGCG), the anticancerogenic polyphenol found in green tea [176, 177], and in neuronal cells exposed to NO [178]. Importantly, Nrf2 is not only an upstream inducer of HO-1, but can be also upregulated in response to HO-1 activation, the effect mediated by CO and PI3K pathway, and this positive feedback loop might be one of potential mechanism of HO-1-induced cytoprotection [179].

Another possible explanation for cytoprotective and antiapoptotic activity of HO-1 is the increase in cellular biliverdin and bilirubin levels. Both compounds are known to inhibit hepatocyte apoptosis *in vitro* [180] and *in vivo* [181]. Bilirubin can also protect keratinocytes against nicotine-induced cytotoxicity [182], and cardiomyocytes against doxorubicin-induced death by increasing Bcl-2 and decreasing Bax expressions [183]. In accordance, supplementation of cultured cells with biliverdin or bilirubin mimicked the cytoprotective action of HO-1, as shown for hepatoma [168] and colon carcinoma [158]. Finally, the effect of HO-1 inhibition on ROS elevation in colon carcinoma was completely abrogated, if cells were supplemented with bilirubin [184]. It must be kept in mind, however, that

unconjugated bilirubin can be toxic, as demonstrated in cervical adenocarcinoma [185] or colorectal cancer [186].

Cytoprotection can be also mediated by HO-1-derived CO [168], which act through activation of both p38-MAPK [130] and sGC [187]. Importantly, CO was not only shown to block the release of mitochondrial cytochrome c, the crucial event in induction of apoptosis, but also to inhibit expression of proapoptotic p53 [164]. Exogenous CO may increase the viability of different cell types, including fibroblasts [101], endothelium [188], hepatocytes [189], and pancreatic β-cells [190]. Like biliverdin, CO can inhibit the doxorubicin-induced apoptosis in cardiomyocytes [183, 191]. However, these effects appear to be cell-type specific, as no CO-exerted protection was found in colon carcinoma, gastric cancer cells, and chronic myelogenous leukemia [78, 158].

Finally ferritin, induced by many HO-1 activators, and then by iron released from heme, can contribute to HO-1 mediated cytoprotection. Indeed, in malignant oral keratino-cytes exposed to NO, HO-1 reduced cell mortality by upregulation of iron regulatory proteins (IRP1 and IRP2), transferrin receptor (TfR), and ferritin [192]. Moreover, susceptibility of tumor cells to oxidants was inversely corre-lated with ferritin protein levels [193]. For example, reduced expression of ferritin resulted in enhanced sensitivity to oxidative stress and apoptosis in melanoma [194] and T-cell lymphoma [195]. Accordingly, increased expression of ferritin was suggested as a mechanism responsible for the protective effect of hemin in leukemia cells subjected to photodynamic therapy [196]. Evaluations of ferritin levels in tumor tissues generated, however, much more complex and perhaps cell-type specific picture: in some tumors, such as colon or breast cancers, ferritin was upregulated when compared with healthy tissues, in others, such as liver cancer, it was decreased [197]. Interestingly, immunohisto-chemical analysis of human melanoma showed that ferritin expression in metastatic lesions was significantly higher than in primary nodules [194].

It must be kept in mind, however, that induction of HO-1 is not always adequate to protect the cells. As demonstrated in breast carcinoma and B-lymphoblasts HO-1 failed to defend the cells from chemotherapy-induced apoptosis [198]. Moreover in some experiments, overexpression of HO-1 enhanced oxidative damage to mitochondria and led to augmented apoptosis in breast cancer cells, astroglia [107], and VSMC [199]. Thus, although HO-1 up-regulation can generally confer antioxidant protection, under certain condi-tions it may promote ROS accumulation within the mito-chondria and other cellular compartments [66]. Nevertheless, the vast majority of studies performed in different tumor cells, such as hepatoma [168], colon adenocarcinoma [87, 158, 168], thyroid carcinoma [122], gastric carcinoma [123], myelogenous leukemia cells [78], and melanoma [108] strongly suggest that HO-1 is a powerful cytoprotective and antiapoptotic enzyme, which improves survival of cancer cells subjected to different therapeutic treatments (Fig. **4**).

ROLE OF HO-1 IN TUMOR ANGIOGENESIS

Angiogenesis, the formation of new blood vessels from preexisting ones, plays a crucial role in growth and spreading of tumors. The notion that tumor progression can be accompanied by increased vascularity was first described in
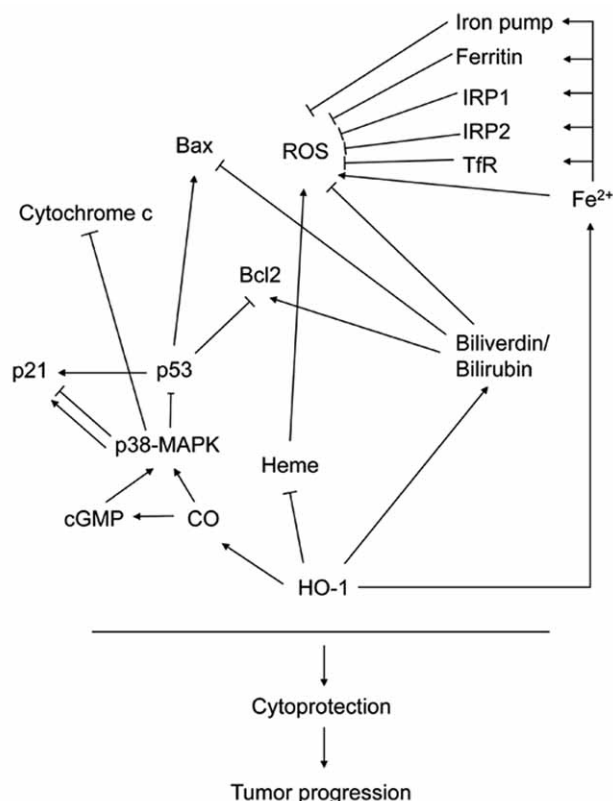
Fig. (4). Schematic demonstration of the role of HO-1 in regulation of cell death in tumors.

the beginning of the 20th century by the German pathologist Rudolf Virchow [200], and then by others [201, 202]. In 1971, a theoretical paper by Judah Folkman postulated that the growth of tumors depends on the induction of new blood vessels, and that inhibitors of angiogenesis might be useful for treating human cancers [203]. Of importance, apart from its cytoprotective actions, HO-1 has been recognized as a proangiogenic enzyme. Thus, one can speculate that these properties may further support the role of HO-1 in tumor progression.

The role of HO-1 in angiogenesis has been shown in different experimental settings. So, endothelial cells isolated from HO-1-deficient mice produced less VEGF than did their wild-type counterparts, and their response to exogenous VEGF and bFGF was weaker [204]. On the other hand, genetic overexpression of HO-1 in endothelial cells inc-reased production of VEGF and facilitated VEGF-induced activities, namely proliferation, migration, formation of capillary-like tubular structures in a Matrigel matrix, and outgrowth of capillaries from endothelial spheroids in a collagen gel [98]. In accordance, HO-1 has been demons-trated to stimulate expression of VEGF also in vascular smooth muscle cells [102] and keratinocytes [205]. At least in microvascular endothelium and vascular smooth muscle cells, this effect appeared to be mediated by CO [98, 125,

206]. Importantly, the results of experiments performed *in vitro* have been confirmed *in vivo* in the rat ischemic hindlimb model or in skin wounds of diabetic mice, where HO-1 gene transfer increased angiogenesis [207].

The influence of HO-1 on tumor vascularization was suggested first by observations that HO-1 expression in tumor infiltrating macrophages correlates with increased vascular density, as shown in human gliomas and vertical growth melanomas [74]. Then it was reported that HO-1 accelerates pancreatic cancer growth by promoting tumor angiogenesis in mice [113]. Significance of HO-1 has been confirmed in experiments where targeted knockdown of HO-1 by siRNA or inhibition of HO-1 activity by SnPPIX impaired the cell survival and VEGF expression, as shown in endothelioma [77], lung carcinoma [114], and in tumors formed by transformed fibroblasts [208]. Moreover, HO-1 inhibition interfered with tumor neoangiogenesis in hepatocellular carcinoma *in vivo* [70]. In concordance, activation of HO-1 in glioma cells with hemin or in breast carcinoma with 15-deoxy-delta-12,14-prostaglandin J_2 (15d-PGJ_2) was accompanied by elevation of VEGF, the effect blocked by pretreatment of cells with HO-1 inhibitor [82]. Finally, in 15d-PGJ_2-stimulated breast cancer cells the expression of matrix metalloproteinase-1 (MMP-1), crucial for growth of capillaries, was HO-1 dependent and facilitated by iron released from HO-1-catalyzed heme degradation [209].

A proangiogenic role of HO-1 was directly evidenced in murine melanoma [108]. Interestingly, HO-1 overexpression in the melanoma cell line did not lead to increased production of VEGF *in vitro*, and VEGF did not contribute significantly to the increased angiogenic potential of media harvested from such cells. However, HO-1 overexpressing melanoma cells inoculated subcutaneously into mice, gave more vascularized tumors with higher content of VEGF, what may suggest an indirect influence of HO-1 present in cancer cells on production of VEGF in stroma, especially in macrophages. This effect could be possibly mediated by diffused CO [108].

It seems that HO-1 might contribute to proangiogenic effects of thymidyne phosphorylase (TP), the enzyme that is often elevated in the blood of cancer patients, and has been implicated in pathophysiological angiogenesis. TP stimulates migration of endothelial cells [210] and formation of capillaries both *in vitro* and *in vivo* [74, 211, 212]. It directly upregulates HO-1, and coexpression of these two proteins was detected in advanced melanoma tumors [74], and bladder carcinoma [211]. TP has been shown to induce oxidative stress in tumor cells, and subsequently the release of angiogenic factors, such as VEGF, IL-8 and MMP-1 [213]. Interestingly, some effects of TP, such as regulation of VSMC proliferation, were blocked both by inhibitors of TP and HO-1 [214].

Similarly, it was found that hepatocyte growth factor (HGF), a multifunctional cytokine of mesenchymal origin [215], promotes hepatocarcinogenesis and stimulates angiogenesis directly or indirectly through upregulation of VEGF [216]. In hepatoma cells it can activate hypoxia-inducible factor-1α (HIF-1α), major proangiogenic transcription factor, *via* stimulation of ROS generation, which induce in turn the HO-1 expression [217]. The major proangiogenic and anti-

apoptotic effects of HGF are similar to that of HO-1. Interrelation of these two pathways may potentially play an important role in tumor progression. This hypothetical supposition needs, however, an experimental verification (Fig. **5**).
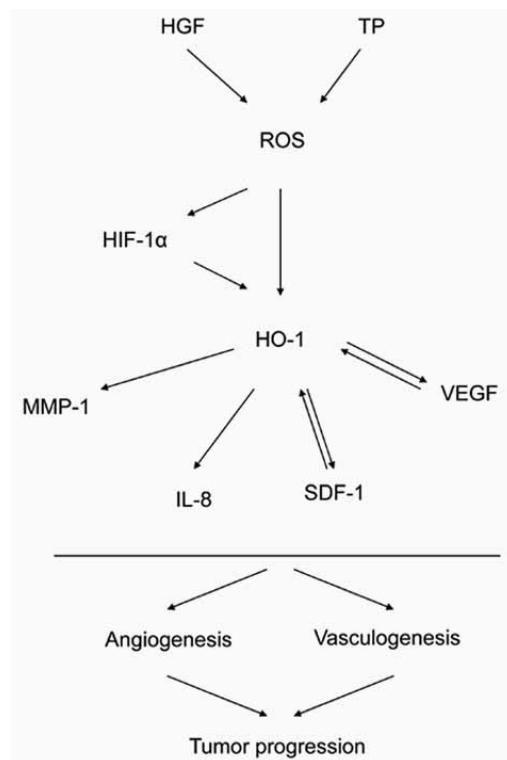

Fig. (5). Schematic demonstration of the role of HO-1 in regulation of angiogenesis in tumors.

Tumor vascularization is a complex process, relying not only on sprouting angiogenesis, but also on adult vasculogenesis, in which bone marrow-derived EPC home to the tumor site, differentiate into mature endothelial cells and incorporate into growing tumor vessels, or stimulate local angiogenesis through production of proangiogenic growth factors [218-220]. One of the key factors governing the EPC homing is VEGF, whose expression and activity is influenced by HO-1. The second very important agent is stroma cell-derived growth factor-1 (SDF-1), a chemokine that plays a major role in migration, recruitment, and retention of EPC to ischemic or injured tissues and contributes to neovascularization.

Analyses of intracranial murine gliomas evidenced the increased production of SDF-1 in the vasculogenic tumors. Furthermore, enforced expression of SDF-1 augmented, whereas blocking the SDF-1 receptor (C-X-C motif receptor-4, CXCR4) reduced homing and engraftment of vascular progenitors [221]. Moreover, SDF-1 may promote the retainment of BM-derived pericytes in close association with perfused, functional tumor vessels. It was convincingly illustrated in experiments, where SDF-1 cDNA was delivered using adenoviral vectors to the tumors in which VEGF was inhibited. SDF-1 overexpression led to augmented tumor growth, increased number of large, lumen-bearing vascular structures, and enhanced vessel pericyte coverage [222]. These observations confirm its role in tumor vascularization.

No data are available on potential contribution of HO-1 in SDF-1-induced activities in tumors. Such an influence, however, is highly possible, in light of recent experiments demonstrating an important role of HO-1 in proper response of endothelial cells or EPC to SDF-1. Thus, SDF-1 activates HO-1 expression both in mature endothelium and in EPC. Pharmacological inhibition or genetic ablation of HO-1 precludes the SDF-1-mediated migration, endothelial tube formation *in vitro* and sprouting of capillaries from aortic rings *ex vivo*, effects that can be restored by CO. The requirement of HO-1 for proper SDF-1 action was confirmed *in vivo* in Matrigel-plug, wound-healing, and retinal ischemia models of angiogenesis and vasculogenesis [223].

Additionally, protection of ischemic myocardium resulting form AAV overexpression of HO-1 correlated with higher production of VEGF and SDF-1, enhanced vascularization, and increased number of progenitor cells recruited to the infarcted area. In accordance, inhibition of VEGF and SDF-1 by neutralizing antibodies significantly attenuated HO-1-mediated neovascularization and protection, evidencing that VEGF and SDF-1 are the downstream effectors of HO-1 activity [224, 225]. Finally, systemic HO-1 induction led to elevated serum levels of VEGF and SDF-1, increased number of circulating EPCs, and improved re-endothelialization of denuded vessels [112]. Therefore, understanding the role of HO-1 in SDF-1-mediated tumor vascularization is urgently needed (Fig. **5**).

ROLE OF HO-1 IN METASTASIS

Angiogenesis is the key event not only in tumor growth but also in metastasis, a multistep process that includes modulation of cell adherence, degradation of surrounding extracellular matrix, migration, proliferation at a secondary site, and neovascularization of newly formed nodules. Metastases spread in three ways - by local extension from the tumor to the surrounding tissues, through the bloodstream to distant sites, or through the lymphatic system to neighboring or distant lymph nodes. The progression of tumor to invasive and metastatic form correlates with poor clinical outcome [226].

Because of proangiogenic properties of HO-1, one can expect that it also would facilitate metastasis. Such a supposition has been confirmed in several models. Namely, melanoma or pancreatic cancer cells engineered to overexpress HO-1 formed much more nodules in the lungs of mice after intravenous injection than their wild-type counterparts [108, 113], whereas pharmacological inhibition of HO-1 completely inhibited metastasis [113]. Accordingly, silencing the HO-1 gene in prostate carcinoma reduced cell invasion *in vitro*, and inhibited growth of primary and metastatic tumors *in vivo* [66]. Finally, clinical data demonstrate that high level of HO-1 expression is associated with lymph node metastasis in oral squamous cell carcinoma patients [227].

However, data on the role of HO-1 in tumor metastasis are inconsistent and may reflect the cell type-specific or development-stage specific effects. For example in MCF-7 breast cancer cell line, HO-1 inhibited the invasion induced by TPA, what resulted from HO-1-dependent decrease in ROS generation, and CO-mediated reduction in MMP-9 expression [228]. Inhibition of MMP-1, MMP-2, MMP-7, and MMP-9 by HO-1-derived CO has also been reported in other cell types [229-231], although MMPs regulation, especially the influence of oxidative stress, depends very much on cellular context [231]. Additionally, in some experiments performed in prostate cancer, the overexpression of HO-1 reduced cell migration and invasiveness *in vitro* [91], in contrast to the other published observations [66]. Finally, in colorectal [233, 234] and oral carcinomas [71, 235, 236], HO-1 was suggested as a marker of lower risk of metastasis. Thus, further studies are necessary to clarify its role. The important and hitherto underestimated aspect is also a distinction the effects of HO-1 expressed in neoplastic and stroma cells.

ROLE OF HO-1 IN TUMOR-RELATED INFLAMMATION

HO-1 is regarded as a potent anti-inflammatory enzyme. Many reports convinced that activation of HO-1 weakens inflammation and may result in immunosuppression [237]. The significance of HO-1 as an immunomodulator has been elegantly illustrated by studies performed in HO-1$^{-/-}$ mice showing that such animals develop progressive inflammatory disease characterized by splenomegaly, lymphadenopathy, leukocytosis, as well as hepatic and renal inflammation [156]. Moreover, HO-1 deficiency lead to strongly enhanced generation of proinflammatory cytokines, including IL-1β, interferon γ (IFNγ), TNF, and IL-6, so in general, Th1-weighted shift in immunity [238].

Many data suggest that overexpression of HO-1 may defend tissues from immune-mediated injury, either through protection against oxidative damage or via local immuno-modulation [47, 61]. The exact mechanisms responsible for anti-inflammatory functions of HO-1 have not been fully elucidated. However, the signaling induced by CO combined with antioxidant properties of biliverdin/bilirubin and ferritin-mediated sequestration of iron could all contribute to this suppression.

CO has been described as an anti-inflammatory mediator in several models of inflammation and tissue injury. Thus, in lipopolysaccharide (LPS)-stimulated macrophages, it decreased the generation of inducible nitric oxide synthase (iNOS)-derived NO and production of TNF, IL-1β, MIP-1β, or IL-6 [61, 239], and these effects were exerted by interfering with AP-1 activity via a c-Jun N-terminal kinase (JNK) pathway [240]. CO reduced also the production of granulocyte macrophage colony–stimulating factor (GM-CSF), which is known to increase the secretion of proinflammatory mediators and promote the maturation of macrophages and neutrophils [241, 242]. Conversely, exposure to CO can increase the synthesis of anti-inflammatory IL-10 both *in vitro* and *in vivo* [61, 231].

Recent data suggest that HO-1/CO may reduce production of proinflammatory cytokines through augmenting the interaction between cav-1 and toll-like receptor-4 (TLR4). Cav-1 inhibits inflammation through direct binding of TLR4, which prevents TLR4 association with MyD88 and TRIF, and blocks downstream activation of NFκB pathway. Cav-1 was also shown to activate p38 MAPK [243], which

was required for translocation of HO-1 to caveolae in LPS-treated macrophages [244] (Fig. **6**).
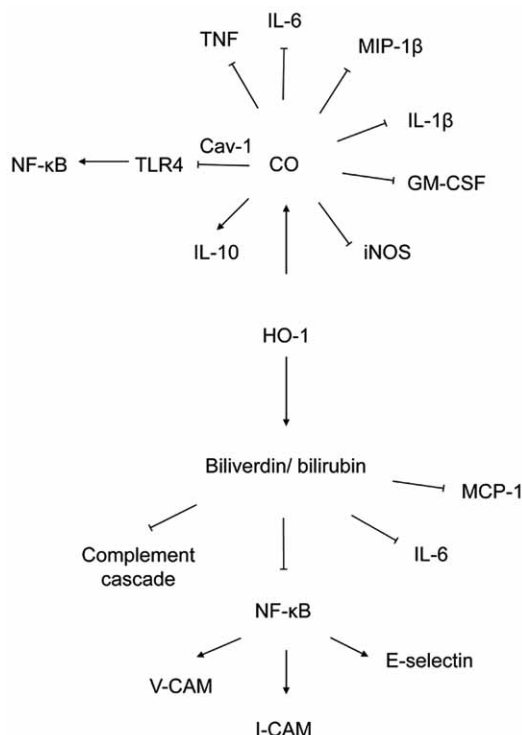

Fig. (6). Schematic demonstration of the role of HO-1 in regulation of inflammatory-related pathways in tumors.

Similarly, inflammatory response can be diminished by biliverdin and bilirubin. For example, they blunt the inflammatory cascade in a model of rodent sepsis [245], and reduce the adhesion of leukocytes to vascular endothelium via inhibition of NFκB-dependent E-selectin, intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) expressions [246]. The importance of biliverdin/bilirubin was also confirmed in HO-1-mediated reduction of monocyte chemotaxis [247]. Interestingly, anti-inflammatory effects of far infrared (FIR) therapy in human endothelium were reversed by pharmacological or genetic inhibition of HO-1, and could be restored by bilirubin [248]. Finally, unconjugated bilirubin can inhibit the initial step in the classical pathway of complement cascade activation, an important effector mechanism of immune system [249] (Fig. **6**).

Tumors are infiltrated by many types of leukocytes, including macrophages, dendritic cells, neutrophils, mastocytes, eosinophils, natural killer (NK) cells, T and B lymphocytes, with macrophages being often the most abundant population [250]. Microenvironmental cytokines may not only selectively promote a Th1 or Th2 immune response, but also polarize macrophages toward the M1 or M2 phenotype [251]. The phenotype of tumor-associated macrophages (TAM) usually resembles that of M2 cells, with pro-tumor functions that include inhibition of Th1 immune response, and production of growth factors, such as epidermal growth factor (EGF), IL-6, IL-8, VEGF, platelet-derived growth factor (PDGF), or transforming growth factor-β (TGF-β). TAM can also induce the degradation and remodelling of extracellular matrix (ECM) *via* the expression of MMPs [252, 253]. In turn, lymphocytes may play a dual role in

tumor progression. Thus, tumor-specific cytotoxic T lymphocytes are able to eliminate tumor cells by direct or antibody-dependent cytotoxicity. However, the recruitment of regulatory T lymphocytes (Treg) attenuates the immune response and may neutralize the cytolytic potential of cytotoxic effectors [251], while tumor microenvironment supports the conversion of naive T lymphocytes into Foxp3$^+$/CD25$^+$ Tregs [254, 255]. It is supposed that activation of Treg cells is one of the main if not the main barrier precluding effective cancer immunotherapies [256, 257].

Inflammation is a key factor in tumor growth and metastasis. Therefore, one can expect that its regulation by HO-1 may affect the progression of tumors. Unfortunately, the experimental data illustrating such an influence are very limited. Interestingly, HO-1 can contribute to macrophage polarization toward M2 phenotype [258] that resembles that of protumorigenic TAMs. It also suppresses the T cell- and NK cell-mediated effector functions [259]. In accordance, elevated HO-1 activity may lead to clonal deletion of CD4$^+$ T cells, resulting in a specific immunomodulation, as illustrated by prolongation of transplanted organ survival [260]. Moreover, it has been demonstrated that HO-1 expression is significantly higher in CD4$^+$CD25$^+$ Tregs than in CD4$^+$CD25$^-$ population [47], and induction of FoxP3 followed by stimulation of regulatory activity in CD4$^+$CD25$^-$ T lymphocytes correlates with HO-1 upregulation [261].

Recent evidence suggests that expression of HO-1 in dendritic cells may further strengthen the tolerogenic effects [262]. It blocks both their maturation and proinflammatory
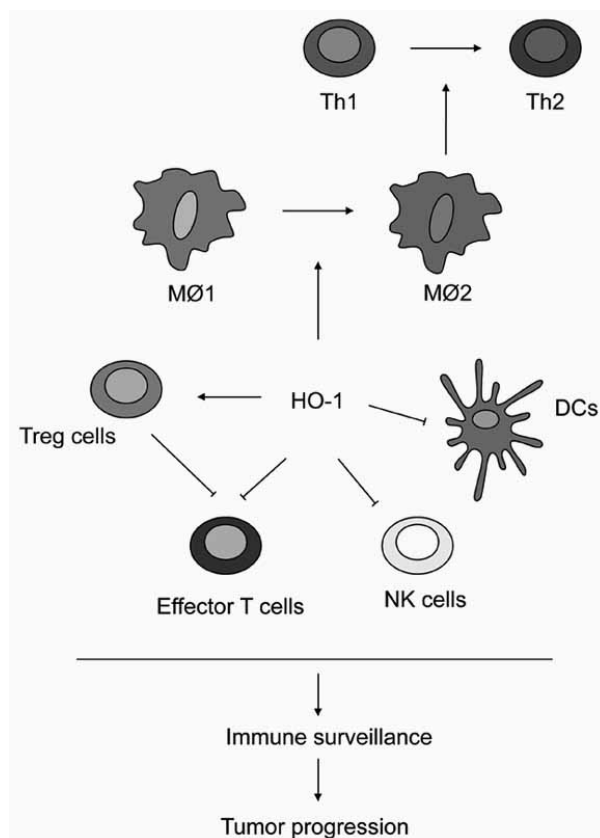

Fig. (7). Schematic demonstration of the role of HO-1 in regulation of actions of inflammatory cells in tumors.

functions but preserves ability to the IL-10 synthesis [231, 263]. Thus, HO-1-expressing dendritic cells may activate suppressor T lymphocytes, thereby boosting suppression of local immune responses. Accordingly, adenoviral transduction of dendritic cells with HO-1 in rats receiving heart transplants led to the long-term allograft survival accompanied by an inhibition of cellular antigraft immune responses [264]. Of interest, naturally occurring CD8^{+} T lymphocytes specific for HO-1 have been shown to suppress cellular immune responses. Such cells were detected *ex vivo* and *in situ* among T lymphocytes from malignant melanoma, renal cell carcinoma, and breast cancer patients. HO-1-specific T cells isolated from the peripheral blood blocked the cytokine release, proliferation, and cytotoxicity of other immune cells. Notably, the inhibitory effect of HO-1-specific T lymphocytes was far more prominent than that of conventional CD4^{+}CD25^{+} Tregs [265]. Thus, HO-1 might be considered as a potential target for accessory strategies facilitating cancer immunotherapies. However, the molecular mechanism(s) responsible for the anti-inflammatory and immunomodulatory effects of HO-1 in tumors have not been elucidated and require further investigations (Fig. **7**).

EFFECT OF HO-1 POLYMORPHISMS IN CANCER PATIENTS

Analysis of clinical data and comparison of HO-1 allele frequency distribution in healthy people and cancer patients can suggest the role of HO-1 in cancer development and anticancer therapy. Importantly, the efficacy of HO-1 pathway can vary in human population due to variable number of (GT)$_n$ repeats, ranging from 11 to 40, in the proximal part of the HO-1 promoter [266]. Shorter fragments of repeats (S alleles) are related to higher transcription rates. It has been shown that lymphoblastoid cell lines derived from human subjects possessing S/S genotype showed much higher activity of HO-1 enzyme after H_2O_2 stimulation than did their L/L counterparts, and only S/S promoters provided protection of lymphoblastoid cells from H_2O_2-induced apoptosis [267]. We have demonstrated that primary human endothelial cells carrying the S allele display a higher expression and activity of HO-1 both in control conditions and in response to some oxidants or inflammatory mediators. Moreover, it is associated with the augmented resistance to oxidative stress, more favorable GSH:GSSG ratio, reduced synthesis of proinflammatory cytokines, and increased cell proliferation [268]. Finally, constitutive expression of HO-1 protein in urothelial cancers was associated with the presence of S alleles [88].

Interestingly, recent reports indicate that lack of short alleles and resulting lower activity of HO-1 can coincide with a higher risk of cancers. The frequency of L alleles was significantly higher in male smokers with lung adenocarcinoma than in control subjects [269]. Furthermore, the longer (GT)n repeats in the HO-1 promoter were linked to the increased risk of areca-induced oral squamous cell carcinoma (OSCC) in males [270]. The frequencies of L-alleles were also significantly higher in esophageal squamous cell carcinoma patients (especially those heavy drinking) than in controls [271]. In turn, the higher risk of breast cancer was observed among postmenopausal women with high iron intake, carrying the HO-1 LL or LM genotypes [272].

Additionally, patients possessing both NFκB ins (resulting in increased activity of the factor) and HO-1 L alleles showed significant risks for various subsets of OSCC, including those of advanced stage with node metastasis [236]. Finally, the long (GT)n sequence in the HO-1 promoter coincided with a higher frequency of gastric adenocarcinoma, whereas the M alleles were associated with a lower frequency of lymphovascular invasion in tumors [273].

However, the effect of HO-1 promoter polymorphism may depend on the type of tumor. For example, the calculated risk for acquiring primary malignant melanoma in L-allele carriers was twofold lower than in those having S/S genotype [274]. In turn, the more active variants of HO-1 and iNOS promoters were associated with increased risk of gastric cancer in women [275].

ROLE OF HO-1 IN CARCINOGENESIS

As mentioned above HO-1 can protect tumor cells against variety of harmful compounds (e.g. oxidants, chemotherapeutics), favoring tumor progression. On the other hand, it inhibits actions of dangerous stimuli, including carcinogens, in healthy tissues. Therefore, the fundamental question is whether HO-1 takes part in healthy - premalignant - malignant transformation or rather blocks this process. To our best knowledge, no studies address the problem directly. Although, it might be speculated that HO-1 could enhance the resistance of cells to carcinogenesis, especially that one induced by ROS or compounds producing ROS, like heme. Genotoxic actions of heme were shown in the human colon cell line and primary human colonocytes [276], whereas carcinogenic only in the colon [277]. On the other hand, antiapoptotic properties of HO-1 could be responsible for preservation of oncogenic mutations and acceleration of tumor transformation.

Importantly, the influence of HO-1 on the expression or activity of oncogenes or cancer-related genes was not elucidated yet. Although, it was shown that HO-1 or its inhibitor, tin mesoporphyrin-IX (SnMPIX) did not affect telomerase and telomerase reverse transcriptase (TERT) [278].

Nevertheless, there are some studies showing that treatment of animals with carcinogens can results in HO-1 induction. Enhanced levels of HO-1 were demonstrated in rats treated with alachlor, the inducer of olfactory mucosal tumors [279]. Correspondingly, the increase of HO-1 was observed in livers exposed to dietary p-dimethylaminoazobenzene (DAB) [65], whereas the downregulation of HO-1 expression was related with malignancy progression [280]. Unfortunately, it is difficult to distinguish whether alteration of HO-1 expression is a reason or outcome of transformation.

Additionally, different levels of HO-1 expression have been detected in the rat strains varying in the vulnerability to carcinogens. For example, carcinogen-resistant DRH rats displaying a significantly lower incidence of liver tumors than the carcinogen-sensitive Donryu strain in response to DAB, have shown much weaker inductions of HO-1 and HGF. Interestingly, these alterations were regarded as an indicator of tissue injury, whereas different levels of expres-

sion of cytochrome P-450 2E, glutathione S-transferase and γ-glutamyltranspeptidase were suggested to be the reason underlying the dissimilar sensitivity of DRH and Donryu rats to carcinogens [281]. Accordingly, the high levels of expression of HO-1 were observed in the Long-Evans with a cinnamon-like color (LEC) rats that spontaneously develops acute hepatitis and hepatoma. Surprisingly, HO-1 overexpression was observed not in cancer lesions but in surrounding healthy tissues, suggesting that HO-1 induction could be rather an adaptive response to oxidative stress [282].

Finally, HO-1 and proliferating cell nuclear antigen (PCNA) expression was correlated with the degree of epithelial dysplasia in human specimen. Interestingly, oral squamous cell carcinoma also showed enhanced expression of HO-1, but this level was not higher than in severe oral epithelial dysplasia (OED) or carcinoma *in situ*. Authors proposed that the augmentation of HO-1 in premalignant oral lesions is part of an early cytoprotection mechanism against carcinogenesis in the oral mucosa [72].

In conclusion, the participation of HO-1 in carcinogennesis needs further investigations, because data available can support both protective and damaging effects. Finally, the discrimination between the primary effect of HO-1 activity on induction of cancer and secondary influence of carcinogenesis on expression of HO-1 is crucial for understanding the problem.

ROLE OF HO-1 IN ANTI-CANCER THERAPY

Studies on the role of HO-1 are important not only for better understanding of tumor-development process but also for clinical practice. Although some reports describe a selective reducing of HO-1 expression in malignant cells, such as adenocarcinoma or tongue squamous carcinoma [235], the majority of analyses indicate that the levels of HO-1 are strongly increased in various tumors [64, 68, 69, 71, 73, 74, 283], especially in those subjected to chemotherapy or exposed to radiation or photodynamic therapy [76, 87, 164] (Table **2**).

This upregulation may have important consequences. In the chronic myeloid leukemia-derived cell line K562,

induction of HO-1 has been shown to counteract imatinib-induced apoptosis [78]. In accordance, high HO-1 expression in pancreatic cancer cell lines was associated with increased chemoresistance to gemcitabine [142]. Similarly, upregulated expression of HO-1 in adenocarcinoma treated with photodynamic therapy led to much faster regrowth of tumors in mice [87]. These results support the idea that HO-1 may be a good candidate in antitumor therapies. Thus, pharmacological inhibition of HO-1 has been suggested as a new therapeutic option and potential sensitizer to chemotherapy, radiotherapy, or photodynamic therapy for chronic myeloid leukemia [78], colon carcinoma [164], adenocarcinoma [87], pancreatic cancer [76], melanoma [108, 167], neoplastic mast cells [109], lung cancer [109], hepatoma [124], and urothelial cancer [88]. The usefulness of such treatments has been confirmed in animal models, as administration of ZnPPIX considerably reduced the growth of hepatoma in rats [69], and sarcoma [284] lung cancer [114] or B-cell lymphoma in mice [124].

However, ZnPPIX is weakly soluble in water, which limits its practical use as a drug. Therefore, PEG conjugation was performed to modify ZnPPIX, resulting in a highly water-soluble complex PEG-ZnPPIX [285]. *In vivo* pharmacokinetic analysis revealed that PEG-ZnPPIX administered intravenously had a much longer plasma residence time, 40 times of nonconjugated free ZnPPIX. In addition, PEG-ZnPPIX preferentially accumulated in solid tumors taking the advantage of EPR (*Enhanced Permeability and Retention*) phenomenon [284]. As a result of EPR effect, macromolecules and lipids (bigger that 40 kDa) preferentially and spontaneously leak out of tumor vessels, and remain there at high concentrations for a long time [286]. Recently, another highly water-soluble micellar form of ZnPPIX was obtained, by the use of amphiphilic styrene-maleic acid copolymer (SMA), named SMA-ZnPPIX. It showed similar HO-1 inhibitory activity as native ZnPPIX and exhibited a potent antitumor potential without any apparent side effects [287].

PEG-ZnPPIX and SMA-ZnPPIX inhibited proliferation and induced apoptosis of canine [288] and human mast neoplastic cells *in vitro* [109], while PEG-ZnPPIX-treated

Table 2. Effects of HO-1 Activation in Tumors

Effect of HO-1 Activity	Type of Cancer
↑Proliferation	Prostate cancer [66], sarcoma [69], hepatoma [69], hepatocellular cancer [70], endothelioma [75], pancreatic cancer [76, 113], angioma [77], urothelial cancer cells [88], melanoma [108], neoplastic mast cells [109], lung cancer [114]
↓Proliferation	Prostate cancer [91], lung adenocarcinoma [110], breast cancer [111]
↑Apoptosis,	Breast cancer [198]
↓Apoptosis	Hormone-refractory prostate cancer [66], hepatoma [69, 70, 168], sarcoma [69, 168], pancreatic cancer [76], chronic myeloid leukemia [78, 165], urothelial carcinoma [88], melanoma [108, 167], thyroid carcinoma [122], gastric carcinoma [123], colon carcinoma [158, 164, 168], lung carcinoma [159, 160], acute myeloid leukemia [162]
↑Angiogenesis	Hepatocellular carcinoma [70], glioma [74], melanoma [74, 108], endothelioma [77], pancreatic cancer [113], lung carcinoma [114], fibrosarcoma [208], breast carcinoma [209]
↑Metastasis	Prostate cancer [66], melanoma [108], pancreatic cancer [113], oral squamous carcinoma [228]
↓Metastasis	Oral carcinoma [71, 236, 237], breast cancer [229], colorectal cancer [234, 235]

colon cancer cells exhibited higher rate of apoptosis [284] and became more vulnerable to hydrogen peroxide, t-butyl hydroperoxide, camptothecin and doxorubicin [164]. These promising results have been confirmed *in vivo*. Namely, treatment with PEG–ZnPPIX significantly reduced the growth of murine sarcoma tumors [284]. Moreover, PEG-ZnPPIX and SMA-ZnPPIX administered to mice allowed to overcome resistance of chronic myelocytic leukemia to imatinib [165]. Finally, it is worth to mention that ZnPPIX or its polymer derivatives PEG-ZnPPIX and SMA-ZnPPIX can also be applied in photodynamic therapies, as porphyrins are known photosensitizers [289]. One important advantage of PEG-ZnPPIX/SMA-ZnPPIX over conventional photosensitizer, is the fact that ZnPPIX sufficiently generates highly reactive singlet oxygen under illumination of visible light and xenon light, not only of laser [287].

It should be kept in mind that pharmacological HO-1 inhibitors, such as SnPPIX, ZnPPIX, zinc mesoporphyrin-IX (ZnMPIX), or zinc deuteroporphyrin-IX (ZnDPIX), as well as HO-1 activators, such as heme, CoPPIX, or CoCl$_2$, are known to exert strong HO-1 independent activities [290, 291]. For example, both ZnPPIX (HO-1 inhibitor) and copper protoporphyrin-IX (CuPPIX, a compound that does not affect HO-1 activity) have been shown to equally diminish tumor blood flow in rats [292, 293], while even a relatively high dose of ZnPPIX (45 μmol/kg) injected intraperitoneally was unable to block the activity of HO-1 in tumors [293]. ZnPPIX induced also an accumulation of ROS in sarcoma [284], colon carcinoma [184], and lung carcinoma [160], causing cell apoptosis, and inhibited expression of cyclin-D1 in colon carcinoma, thereby decreasing the rate of proliferation [184]. Again, these effects seemed to be HO-1 independent. Finally in hepatoma and chronic myelogenous leukemia cells, the inhibition of proliferation by ZnPPIX could not be mimicked by SnPPIX, indicating the HO-1 independence [124].

Much more specific strategy is the employment of siRNA. Experiments performed *in vitro* and in animal models clearly confirm that HO-1 could be a promising target of anticancer therapy. For example, targeted knockdown of HO-1 achieved by transfection of siRNA induced apoptosis of cultured colon carcinoma [158, 284] or leukemic cells [162, 165], and resulted in diminished proliferation, reduced growth and decreased angiogenesis in orthotopic hepatocellular tumors [70]. The same approach in prostate cancer cells led to reduced cell proliferation, decreased cell survival, and attenuated cell invasion *in vitro*, what was reflected by inhibition of prostate tumor growth and lymph node and lung metastases *in vivo* [66]. Similarly, HO-1 siRNA increased apoptosis, augmented the cisplatin cytotoxicity toward lung cancer cells [85, 160], and sensitized pancreatic carcinoma to oxidative stress, gemcitabine, or γ-radiation [76].

The RNAi approach has been widely used for drug development and several clinical trials are ongoing [294]. However, there are still some concerns and challenges to be overcome, such as efficacy and specificity of oligonucleotide delivery, potential off-target effects, and activation of innate immune responses. Various siRNA delivery strategies have been established, including employing of nanoparticles, cationic lipids, antibodies, cholesterol, aptamers and viral vectors for short hairpin RNAs (shRNAs). In some cases the oral or aerosol-mediated delivery might be more favorable. For example in mice, the aerosol delivery of anti-Akt1 siRNA was successfully carried out to treat the lung cancer [295].

Ectopically applied siRNAs can modify the expression of non-targeted transcripts [296, 297]. Thus, it has been shown that inhibition of neovascularization by siRNA in murine model of age-related macular degeneration was not due to a specific knockdown of VEGF and its receptor, but due to a dsRNA-dependent activation of TLR3, triggering the IFNγ and IL-12 production [298]. Moreover, certain motifs in siRNAs can trigger the type I IFN production via activation of TLR7 and TLR8 [299, 300]. Fortunately, this off-targeting can be controlled by a 2'-OMe modification of siRNA [301]. It should be also kept in mind that the levels of siRNA have to be controlled, as too high expression in studies with AAV vectors, where Pol III was used to express shRNA, led to in lethality of mice due to acute liver failure [302]. Thus, the siRNA strategy could be a promising tool for specific and targeted HO-1 inhibition in cancers.

CONCLUSIONS

Protumoral activities of HO-1 seem to be related to its cytoprotective and antiapoptotic properties, which result in better tumor cell survival and higher resistance to different types of therapies. HO-1 affects also tumor cell proliferation and differentiation, which may lead to development of more aggressive phenotype. Moreover, HO-1 can facilitate angiogenesis, a key process in growth of primary and secondary tumors. Finally, HO-1 modulates behavior of inflammatory cells, favoring state of immunosupression that assists tumor progression. Thus, HO-1 seems to accelerate tumor growth and metastasis. Therefore, we propose that inhibition of HO-1 activity might be considered as an independent therapeutic strategy or as a sensitizing approach in combination with chemotherapy, radiation, or photodynamic therapy to potentiate their anticancer effects (Fig. **8**). On the other hand, HO-1 can protect healthy tissues against carcinogenesis, and can be beneficial if expressed in stroma cells. Consequently, it seems that such inhibition should be targeted specifically to the cancer cells.
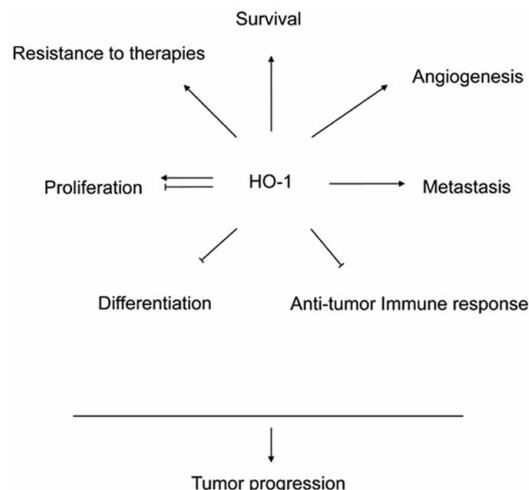

Fig. (8). Schematic summary of HO-1 effects on tumor biology.

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