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DIVERSE ACTIONS OF HYPOXIA-INDUCIBLE FACTOR 1 INHIBITORS IN CANCER

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ABSTRACT

In human cancers, hypoxic mass developed upon the quick tumor expansion has been considered as a driving force of hypoxia inducible factor 1 (HIF-1) mediated cancer progression. Moreover, hypoxic microenvironment has been an impediment for the available conventional therapy causing resistance to chemotherapy as well as radiotherapy. This has led to the development of small molecules targeting hypoxic tumor microenvironment, specifically to inhibit HIF-1 mediated signaling. In this review, we emphasize on different small molecules developed to target hypoxia signaling and their proposed mechanism of actions.

KEYWORDS:

INTRODUCTION

Cancer cells experience different gradients of oxygen as they grow and expand in to mass and consequently evolve certain survival strategies to adapt to such hostile environment. Among all the survival pathways activated in cancer cells, HIF-1 mediated survival has gained importance as it is responsible for therapy resistance and subsequent poor patient prognosis. Treating cancer cells alone without affecting the normal cells has been a difficult task and this led to the development of therapies targeting the signaling pathways that are deregulated only in cancer cells. Considering the fact that HIF-1 mediated cell signaling varies among normal cells and hypoxic cells in a tumor microenvironment, scientists have designed and developed certain small molecules to inhibit hypoxic signaling.

An ever increasing number of agents are constantly being reported that inhibit HIF-1 signaling. Based on the collective reports available so far on the HIF-1 inhibitors, they can be simply classified in to agents that act by modulating HIF-1α mRNA expression, Protein translation of HIF-1α, Protein degradation HIF-1α, DNA binding of HIF-1α and Transcriptional activity of HIF-1α.[1] In this review, we will discuss about different HIF-1 inhibitors developed including their mechanism of action.

HIF-1α inhibitors acting through various mechanisms

EZN-2968

EZN-2968 is an antisense oligonucleotide, developed to target specific HIF-1α inhibition.[2] EZN-2968 is highly specific to HIF-1α and binds HIF-1α mRNA with high affinity resulting in its down-regulation and subsequent reduction in the levels of HIF-1α protein, both *in-vitro* and *in-vivo*. As per the literature, treatment with EZN-2968 exhibited inhibition of tumor cell growth and down-regulation of HIF-1α target genes in HUVEC *in-vitro*. *In-vivo* administration of EZN-2968 decreased endogenous mRNA levels of HIF-1α and vascular endothelial growth factor (VEGF) in the liver of normal mice. Also, antitumor activity has been reported in human prostate cancer (DU145) xenograft models. Preliminary outcome of ongoing phase I clinical trials in patients with advanced solid tumors show that EZN-2968 can be given safely and potential activity of the molecule has been observed in one patient with metastatic renal cell carcinoma (RCC).

Aminoflavone

Aminoflavone is another agent known to affect the expression of HIF-1α mRNA. It is a ligand of the aryl hydrocarbon receptor (AhR). It has been in phase I clinical trials in patients with metastatic cancer. It is believed that AhR dimerizes with HIF-1α and this created interest to examine whether the HIF-1α levels are affected by the pharmacological activation of AhR pathway using AF. However, results of these studies have proven that Aminoflavone does inhibit the accumulation of HIF-1α, and thus, the proposed mechanism of HIF-1 inhibition by Aminoflavone is the modulation of HIF-1α mRNA expression, although the exact mechanism remains to be completely elucidated.

Topotecan

Topotecan is one of the first FDA approved chemotherapeutic agents described that may affect translation of HIF-1 protein and is currently used as second-line therapy for patients with small cell lung cancer and ovarian cancer. Despite the fact that the basic mechanisms

underlying hypoxic regulation of HIF-1translation are inadequately understood, several agents have been reported which may affect the rate of HIF-1protein synthesis. These agents consist of the topoisomerase I and II inhibitors[3-5], receptor tyrosine kinase inhibitors[6-8], cyclin-dependent kinase inhibitors[9], inhibitors of oncogenic pathways[10-14], thioredoxin reductase inhibitors[15], activators of p53[16] and microtubule disrupting agents.[17] Topotecan is a camptothecin analogue that was originally identified in a high throughput screen using a cell-based assay of HIF-1 transcriptional activity at the National Cancer Institute.[18] It induces the formation of stable Top I-DNA cleavage complexes and generates double strand DNA breaks and cytotoxicity during DNA replication and thereby poisoning Topoisomerase I. Interestingly, topotecan found to inhibit the translation of HIF-1 by a Top1-dependent mechanism without any DNA damage. This suggests that both Cytotoxicity and HIF-1inhibition by Topotecan could be mechanistically distinguished.[19] In fact, low-dose administration of Topotecan on a daily basis in a mouse xenograft glioma model showed inhibition of HIF-1protein level expression, angiogenesis and tumour growth.[20] In recent studies, it has been shown that daily administration of Topotecan in combination with the anti-VEGF antibody Bevacizumab exerted synergistic antitumour activity in xenograft models, which provides a rationale for clinical development of this combination strategy.[21] A pilot study is planned and ongoing at NCI in which daily oral topotecan is given to patients with metastatic refractory cancers so as to provide evidence whether this agent is able to affect HIF-1 signalling in tumour tissue. As topotecan has a short biological half-life, it is likely that other topoisomerase 1 inhibitors with more favorable pharmacokinetics may be more suitable for chronic suppression of the HIF-1 pathway. In this regard EZN-2208 is an interesting agent which is a PEGylated form of SN38, the active component of CPT-11 (Irinotecan Pfitzer, New York, NY, USA; Yakult Honsha, Tokyo, Japan), described by better pharmacokinetics and by significant antitumour activity in pre-clinical models of solid tumours and lymphomas, including CPT-11-resistant tumours [Sapra 2008]. The established activity of EZN-2208 in CPT-11 resistant tumours could be potentially explained by the ability of this agent to inhibit HIF-1accumulation, thus acting on the tumour microenvironment rather than only on cancer cells.[5] EZN-2208 is currently in phase I and phase II clinical trials, as well as combination studies were planned.

Digoxin

Another class of agents that have been reported recently to affect HIF-1protein translation is the Cardiac glycosides. Among different agents of this class, in particularly, Digoxin was

identified as a potent HIF-1 activity inhibitor in a cell-based screen of a chemical library of FDA approved agents.[22] Digoxin was shown to inhibit the translation of HIF-1 by an mTOR-independent mechanism and exerted significant antitumour activity in xenograft models. Interestingly, digoxin, which is normally used for the treatment of heart failure and cardiac arrhythmias, is currently being tested in a phase I clinical trial as a potential anticancer agent. Fascinatingly, digoxin suppressed tumour growth in PC3 and P493-Myc tumour xenografts, however did not affect the growth of xenografts expressing a constitutively active form of HIF-1 i.e HIF-1beta, implicating HIF-1alpha in the antitumour activity of digoxin. Consistent with these results, fractionation of an extract of the plant *Crossosoma bigelovii* led to the discovery of a new strophanthidin glycoside which also inhibited transcriptional activity of HIF-1.[23] Whether cardiac glycosides may effectively be used to inhibit HIF-1 in cancer patients without any unacceptable adverse events, remains to be established.

PX-478

PX-478 is another HIF-1 inhibitor which is currently in phase I clinical trials in patients with advanced metastatic cancer. This agent showed remarkable antitumour activity in a variety of human tumour xenograft models, which seemed to correlate with levels of HIF-1 expression.[24] PX-478 inhibited constitutive and hypoxia-induced HIF-1expression in a pVHL and p53 independent manner. The inhibition seems to occur at numerous levels, as three different mechanisms have been proposed that might contribute to the decreased levels of HIF-1accumulation. In fact, it has been suggested that PX-478 inhibits HIF-1 deubiquitination, leading to augmented degradation of polyubiquitinated HIF-1, diminishes the expression of HIF-1 mRNA and also affects translation of HIF-1.[25] Results of an ongoing phase I trial might provide remarkable information regarding antitumour activity and regulation of HIF-1levels in cancer patients.

mTOR is a signaling pathway, implicated in growth factor- dependent induction of HIF-1 translation.[26] However, global protein synthesis and mTOR are inhibited under severe hypoxic conditions and thus the contribution of these pathways to HIF-1 translation under such conditions is still poorly understood.[27] Several mTOR inhibitors, including temsirolimus and everolimus (that are FDA approved agents for the treatment of renal cancer) have been shown to inhibit HIF-1.[28-29] Clinical trials have demonstrated efficacy of these agents in the treatment of RCC.[30] Clinical trials are ongoing to assess the potential of

mTOR inhibitors, as single agents or in combination, for the treatment of other solid malignancies. Hence, whether inhibition of HIF-1 contributes to the therapeutic activity of this class of agents in solid malignancies other than renal cell carcinoma remains to be established.

Hsp90 inhibitors

Hsp90 is a molecular chaperone which controls the folding of different proteins and regulates their function as well. Those proteins include receptor tyrosine kinases, serine/threonine kinases, transcription factors and activated oncoproteins.[31] HIF-1protein stability is also affected by its interaction with Hsp90. In the presence of Hsp90 inhibitors HIF-1undergoes VHL-independent proteasomal degradation[32]; moreover HIF-1 heterodimers may not acquire the proper conformation and fail to recruit cofactors important for HIF-1-mediated transcriptional activity.[33] The development of Hsp90 inhibitors started with the discovery of the natural product geldanamycin, a benzoquinone ansamycin antibiotic that inhibits Hsp90 by competing with the ATP binding site. Geldanamycin was found to induce HIF-1 degradation under both hypoxic and normoxic conditions in several cell lines. 17-AAG and 17-DMAG are the first Hsp90 inhibitors to enter clinical trials and currently, a hefty number of second generation Hsp90 inhibitors are under clinical development as anticancer agents.[34] However, given the range of client proteins that may be affected by Hsp90 inhibition, it is hard to determine whether and to what extent their antitumor activity may be related to HIF inhibition, more so in the absence of clinical trial aimed at assessing the definite effect of Hsp90 inhibition on HIF-1 signalling pathway. Histone deacetylase inhibitors have also been shown in the regulation of HIF-1 activity by several potential mechanisms, including induction of HIF-1protein degradation and regulation of HIF-1 transcriptional activity.[35] Although a direct role of acetylation in the regulation of HIF-1protein remains controversial, current evidence shows that Sirtuin 1 (Sirt1) which is a redox-sensing deacetylase, selectively stimulates activity of HIF-2during hypoxia.[36] Histone deacetylase inhibitors are currently evaluated in a number of solid tumours either as single agents or in combination and inhibition of HIF-1 should be considered as a potential mechanism contributing to their activity.

Echinomycin

Inhibition of HIF-1 DNA binding to the hypoxia responsive element (HRE), a step required for induction of transcription, is also a potential mechanism by which small molecules may

inhibit HIF-1 activity.[37-38] Proof of principle that this mechanism may effectively inhibit HIF-1 transcriptional activity was provided by the identification of echinomycin, a cyclic peptide of the family of quinoxaline antibiotics originally isolated from Streptomyces echinatus, which was known to bind DNA in a sequence-specific fashion. Echinomycin was shown in chromatin immunoprecipitation experiments to inhibit HIF-1, but not AP-1 or NF-κB, binding to DNA, providing evidence of a fairly selective inhibition based on recognition of DNA sequences. Echinomycin clinical development was halted in the late 1980s following extensive testing as cytotoxic agent in phase I-II trials, which failed to show significant activity.

Anthracyclines

In recent times, anthracyclines were found to inhibit HIF-1 activity.[39] Anthracyclines exert their cytotoxic activities by a number of different mechanisms, including DNA intercalation. These are among the most efficient chemotherapeutics used to treat a variety of cancers. Recently evidence showed that daunorubicin and doxorubicin inhibit HIF-1 transcriptional activity by blocking its binding to the HRE sequence. In mice bearing human prostate cancer xenografts, administration of daunorubicin or doxorubicin significantly repressed tumour growth and vascularization, along with a drop off in circulating angiogenic cells (CAC). The movement of CACs in the bloodstream was shown to be mediated by HIF-1-induced genes which encode pro-angiogenic cytokines that found to be significantly down-regulated in mice treated with anthracycline. These results increase the possibility that metronomic administration of anthracyclines may possibly exert antitumour activity by inhibiting HIF-1 and angiogenesis.

Chetomin

Chetomin is an epidithiodiketopiperazine metabolite of the fungal species *chaetomium*. It was found to inhibit HIF-1 activity by disrupting the interactions of c-terminal transactivation domain (CTAD) of HIF-1α protein with the CH-1 domain of p300 which is a transcription coactivator.[40] Thus chetomin reduces the formation of HIF-1α/p300 complex thereby attenuating hypoxia-inducible gene expression. Some studies revealed that in human HT1080 cells, inhibition of HIF-1α by chetomin significantly reduced mRNA expression of CA9 and VEGF and thus improved response to radiation therapy under severe hypoxic conditions.

Bortezomib

Bortezomib (PS-341) is a proteasome inhibitor. It is an FDA approved drug for treatment of multiple myeloma and mantle cell lymphoma.[41-42] Interestingly, its antitumour activity may correlate with its ability to suppress transcriptional activity of HIF-1.[43] Bortezomib was found to impair the p300-HIF-1interaction, by enhancing the binding of FIH to HIF-1 at low nanomolar concentrations.[44] fascinatingly, the doses of bortezomib that inhibit HIF-1activity are found to be much lower than those required to inhibit proteasome function and this suggests that the HIF inhibition by bortezomib may be independent from Proteasome inhibition.[44]

Chrysin

Chrysin (a natural flavonoid) has been shown to possess anticancer effect but the mechanisms are not clearly establishd. Studies on the effect of chrysin on HIF-1α and vascular endothelial growth factor (VEGF) expression in DU145 cells (human prostate cancer cells) showed that chrysin inhibited expression of HIF-1α degradation induced by insulin through ubiquitination by increasing its prolyl hydroxylation. It also interfered with interaction between HIF-1α and HSP90. In addition, chrysin was found to inhibit HIF-1α expression through Akt signaling. Some studies showed that chrysin inhibited DU145 xenograft induced angiogenesis in nude mice. Thus chrysin has shown to inhibit the expression of HIF-1α by reducing its stability and inhibiting its protein synthesis which suggests that it is a potent inhibitor of HIF-1α and provide a new insight into the mechanisms of chrysin against caners.[45]

Silibinin

Silibinin is a non-toxic flavonoid which has been reported to exhibit anticancer activity; however, the mechanism is not well established. In HeLa and Hep3B cells, it was found to inhibit accumulation of HIF-1α induced by hypoxia and its transcriptional activity and this was correlated with strong dephosphorylation of mTOR and its effectors p70S6K (ribosomal proteinS6 kinase) and eukaryotic initiation factor 4E-binding protein-1 (4EBP1). This mTOR/p70S6K/4E-BP1 pathway is known to regulate HIF-1α expression at its translational level. Silibinin has also shown to reduce hypoxia-induced VEGF release by HeLa and Hep3B cells. Thus these results suggest silibinin as an effective inhibitor of HIF-1 and present new approaching into the mechanism of its anticancer activity.[46]

YC-1

YC-1 is a novel antitumor agent, found to inhibit HIF-1α accumulation in response to hypoxia. It was found to suppress PI3K/Akt/mTOR/4E-BP1 pathway which regulates the translation of HIF-1α but it did not affect either mRNA levels of HIF-1α or its half life.[47] Studies demonstrated that YC-1 inhibited the activation of NF-κB in response to hypoxia which suggests that NF-κB contributes to Akt-mediated HIF-1α accumulation during hypoxia. YC-1 has been shown to inhibit HIF-1α and HIF-1α-mediated gene expression like VEGF, MMP-2, Bcl-2, cell proliferation and migration activities and also induced apoptosis in hypoxic human bladder transitional carcinoma cell line T24 cells (BTCC) in a dose-dependent manner. The possible mechanism responsible for YC-1 mediated HIF-1α suppression may be the ERK/p38 MAPK pathway.

Q39

Q39 is a quinoxaline, 1, 4-Di-N-oxide derivative i.e. 3-(4-bromophenyl)-2-(ethyl-sulfonyl)-6-methyl quinoxaline-1,4-dioxide. It has been reported to exhibit significant antiproliferative avtivity against hepatoma cells in normoxia and hypoxia (both *in-vitro* & *in-vivo*), mainly by inducing apoptosis. It also resulted in a drastic decrease in VEGF expression due to the suppression of HIF-1α. It did not show any affect on the degradation rate of HIF-1α or on the steady-state levels of HIF-1α mRNA, but the reduction of HIF-1α accumulation by Q39 was correlated with prominent dephosphorylation of mTOR and 4E-BP1.[48]

Tirapazamine

Tirapazamine is a bioreductive anticancer agent which entered Phase III clinical trials. It is shown to exhibit anticancer activity by inhibiting topoisomerase IIα and for the first time in 2010, studies conducted by Jun Zhang et al. showed that the compound acts in a novel manner to inhibit HIF-1α accumulation in response to hypoxia in human cancer cells and in HepG2 cell-derived tumors in athymic nude mice. Their studies in HeLa cells demonstrated that Tirapazamine was involved in the regulation of HIF-1α translation. Further studies revealed that the inhibitory effect of HIF-1α protein synthesis is depedndent on the phosphorylation of eukaryotic initiation factor 2α (eIF2α) rather than the mTORC1/4e-BP1 pathway. Moreover, immunofluorescence analysis suggested that HIF-1α inhibitory activity of the drug molecule is independent of its topoisomerase IIα inhibition. Hence, these findings suggest Tirapazamine as a potent regulator of HIF-1α and provide new insight into the potential molecular mechanism whereby it serves to reduce the expression of HIF-1α.[49]

6α-Tigloyloxychaparrinone (TCN)

6α-tigloyloxychaparrinone is a quassinoid obtained from the tree *Aliantus altissima*. It was found to inhibit HIF-1 pathway by inhibiting the phosphorylation of eIF4E. The effect of TCN on hypoxia or $CoCl_2$ induced HIF-1 activation has been investigated and it showed potent inhibitory activity against HIF-1 activation in various human cancer cell lines. TCN was shown to decrease the hypoxia-induced accumulation of HIF-1α in a dose-dependent manner and it also suppressed the expression of HIF-1 target genes for vascular endothelial growth factor (VEGF) and erythropoietin (EPO) in response to hypoxia. Further analysis revealed that the molecule strongly inhibited HIF-1α protein synthesis without affecting the expression level of HIF-1α mRNA or degradation of HIF-1α protein. In addition, the phosphorylation levels of extracellular signal-regulated kinase-1/2 (ERK1/2), mitogen activated protein (MAP) kinase-interacting protein-1 (MNK-1) and eukaryotic initiation factor 4E (eIF4E) were significantly suppressed by TCN treatment, without altering the levels of these proteins. Thus 6α-tigloyloxychaparrinone could be a new HIF-1 targeted anticancer agent and be effective on mTOR-targeted cancer therapy in which mTOR inhibition increases eIF4E phosphorylation.[50]

P3155

P3155, a pirydylpyrimidine derivative is a novel inhibitor of HIF-1α. It was also found to modulate PI3K pathway thus inhibiting the growth of prostate cancer cells.[51] Studies revealed that down regulation of HIF-1α protein by P3155 is independent of ubiquitin-proteasomal pathway and also induced degradation of HIF-1α protein in a dose-dependent manner with no effect on HIF-1α by P3155 may be at its translational level. In hypoxic PC3 cells, treatment with P3155 resulted in reduced expression of HIF-1α as well as decreased phosphorylation of Akt and its downstream effector 4E-BP1 in a dose-dependent manner.[52] Immunofluroscence studies demonstrated the abrogation of HIF-1α and VEGF expression by P3155. Wound healing assays of P3155 molecule were performed to study the cell migration, polarization as well as proxy for angiogenesis and metastasis.[53] The results suggested that the molecule inhibits cell migration and angiogenesis via inhibition of HIF-1α and PI3K/Akt signaling pathway. These promising *in-vitro* results lead to the examination of tumor growth inhibition due to P3155 in prostate cancer when given by per oral route. It showed significant anti-tumor activity with 61% growth inhibition. Thus this agent can be used as a therapeutic modality for aggressive prostate cancer.

CL67

CL67 is a DNA quadruplex-stabilizing disubstituted naphthalene derivative. In renal carcinoma cell lines and in mouse xenograft model of renal cancer, CL67 was identified as a potent inhibitor of the HIF-pathway and the proposed mechanism is that it found to act by binding to and stabilizing G-quadruplex structures in HIF-1α leading to decreased transcription, downregulation of HIF-1α protein and inhibition of HIF transactivation with subsequent reduction in expression of downstream target genes, both *in-vitro* and *in-vivo.* Therefore, CL67 symbolize an exciting therapeutic agent with a novel mechanism of action that may offer therapeutic advantage in the treatment of human renal cancers.[54]

2-Methoxyestradiol

2-Methoxyestradiol is a natural metabolite of estrogen, shown to inhibit the activation of HIF-1alpha through destabilization of microtubules and has antiproliferative and proapoptotic effects upon various cancerous cells *in-vitro.* It has shown anticarcinigenic activities in several preclinical models of cancer. Evaluation of 2-Methoxyestradiol in clinical trials for the treatment of multiple myeloma, advanced solid tumors, prostate & metastatic breast cancer was initiated in 2004. Justin et al. in 2004, investigated the effects of 2-Methoxyestradiol alone and in combination with paclitaxel where in 2-Methoxyestradiol exhibited antiproliferative and cytotoxic effects in a panel of five head & neck squamous cell carcinoma cell lines, including the induction of G2-M blockade, caspase-3, caspase-7 activation and apoptosis at 48h.[55] It resulted in decreased nuclear HIF-1alpha binding activity and affected the expression of downstream genes like bid – a proapoptotic bcl-2 family member and VEGF – a proangiogenic cytokine. It alo exhibited antitumor and antiangiogenic activity, *in-vivo,* in a xenograft model of head & neck squamous cell carcinoma using UM-SCC-11A cells. Thus, these results provide support for the treatment of recurrent or advanced head & neck squamous cell carcinoma.

In 2008, Wanqiu Chen et al. studied the effects of 2-Methoxyestradiol in neonatal brain damage after hypoxic-ischemic injury. They tested 2-Methoxyestradiol at different doses by administrating 2-Methoxyestradiol immediately and 3h after induction of hypoxic-ischemic injury. The results revealed that 2-Methoxyestradiol exhibited dose-dependent neuroprotection by decreasing infarct volume and attenuating brain edema. It was also found to attenuate blood brain barrier disruption when examined by IgG staining and decreased the

expression of HIF-1alpha and VEGF which are studied by immunohistochemistry and western blotting analysis.[56]

CONCLUSION

Better understanding of hypoxic tumor microenvironment and HIF-1 signaling laid emphasis on developing small molecule inhibitors for HIF-1. However, lack of specificity is the most common limitation of most of the HIF-1 inhibitors as they inhibit multiple targets. Hence small molecules should be validated for HIF-1 inhibition using specific models and efforts should be made to develop a promising standard molecule that could effectively downregulate hypoxic signaling in cancer cells.

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