Review

Drug Testing and Analysis

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Hypoxia-inducible factor stabilizers and other small-molecule erythropoiesis-stimulating agents in current and preventive doping analysis

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Increasing the blood's capacity for oxygen transport by erythropoiesis-stimulating agents (ESAs) constitutes a prohibited procedure of performance enhancement according to the World Anti-Doping Agency (WADA). The advent of orally bioavailable small-molecule ESAs such as hypoxia-inducible factor (HIF) stabilizers in the development of novel anti-anaemia therapies expands the list of potential ESA doping techniques. Here, the erythropoiesis-stimulating properties and doping relevance of experimental HIF-stabilizers, such as cobaltous chloride, 3,4-dihydroxybenzoic acid or GSK360A, amongst others, are discussed. The stage of clinical trials is reviewed for the anti-anaemia drug candidates FG-2216, FG-4592, GSK1278863, AKB-6548, and BAY85-3934. Currently available methods and strategies for the determination of selected HIF stabilizers in sports drug testing are based on liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). For the support of further analytical assay development, patents claiming distinct compounds for the use in HIF-mediated therapies are evaluated and exemplary molecular structures of HIF stabilizers presented. Moreover, data concerning the erythropoiesis-enhancing effects of the GATA inhibitors K7174 and K11706 as well as the lipidic small-molecule ESA PBI-1402 are elucidated the context of doping analysis. Copyright © 2012 John Wiley & Sons, Ltd.

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Introduction

Haemoglobin (Hb) containing red blood cells (RBCs) are the major haematological factor controlling the organism's capacity for the uptake, transport, and delivery of oxygen to energy-consuming tissues. Since the performance of exercising muscles is directly dependant on the amount of O_2 available for respiratory energy supply, an increase in RBC mass eventually corresponds to an elevated aerobic stamina.[1] While the effect of increasing the blood's potential for oxygen delivery is salutary in the treatment of diseases with pathologically decreased RBC count (anaemia), an artificial manipulation of this haematological parameter constitutes an illicit method of performance enhancement in sports. According to the World Anti-Doping Agency (WADA), blood doping techniques like autologous or homologous blood transfusion (including the transfer of RBC products, WADA category M1.1), methods that artificially enhance the capacity of oxygen transport (category M1.2) as well as the use of erythropoiesis stimulating agents (ESA, category S2.1) are prohibited in sports.[2] The effective use of recombinant human erythropoietin (rhEPO) in the ESA-based therapy of anaemic disorders (e.g. related to chronic kidney disease, CKD, cancer, or chemotherapy) has arguably enforced the widespread misuse of rhEPO and numerous biosimilars as doping agents. In addition, the progress in understanding the physiologic regulation of the erythropoietic system made in the last ten years has revealed new pivot points for a pharmacological manipulation. Besides the challenges of detecting traditional protein-based ESA and blood doping techniques,[3–5] the discovery of small molecules that stimulate the erythropoietic cascade by mechanisms more complex than the sole action of EPO as a growth factor, will soon add a new facet to the complex blood doping. This review presents the recent progress in the research and development of small molecule ESA therapies, with a focus on the molecular structures of potential therapeutic agents, the doping relevance of yet unapproved substances and their possible direct detection in sports drug testing. Due to the plethora of published data in this field, an emphasis is placed on the discussion of the hypoxia-inducible factor (HIF) pathway and its pharmacological manipulation but other emerging small-molecule ESAs such as GATA-inhibitors (*vide infra*) are also covered.

HIF stabilizers

HIF signalling

The hypoxia-inducible transcription factor is the key regulator for the body's systemic and local response to oxygen deprivation. It

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was first identified in 1992 by Wang and Semenza to bind to the hypoxia-responsive element (HRE) in the enhancer region of the erythropoietin gene.[6] The transcriptional activator regulates physiological processes such as erythropoiesis, neovascularisation, and oxidative metabolism of the cell and its pivotal role in the course of various diseases has made it an important target for pharmacological manipulation.[7] Besides the intentional therapeutic purpose of HIF regulation, a glance at the physiological parameters controlled by this system also alerts anti-doping authorities.[8]

HIF is a heterodimeric protein composed of an α- and β-subunit.[9] Both units are constitutively expressed and, while HIFβ is usually present in excess compared to the α-subunit, the stability of the latter strongly depends on tissue oxygen concentration. In normoxia, post-translational hydroxylation of distinct amino acid residues in the HIFα subunit leads to its inactivation. Prolyl hydroxylation allows binding to the von Hippel-Lindau protein (pVHL) E3 ubiquitin ligase complex, which leads to rapid proteasomal degradation. Under hypoxic conditions, the proline modification is suppressed, leading to stabilization and accumulation of HIFα in the cytosol. Translocated into the nucleus, it dimerises with the HIFβ subunit and the co-activator complex p300/CBP, binds to the HRE and promotes expression of HIF target genes (e.g. EPO, for review see references[10,11]).

Three different HIFα isoforms have been identified to date, with HIF-1α being the most extensively studied isoform. It is ubiquitously expressed, whereas HIF-2α displays a more restricted tissue distribution.[12] While both isoforms are induced under hypoxic conditions, HIF-3α was proposed to act as a negative regulator of hypoxia-inducible cell responses.[13] Although HIF-1α and HIF-2α share a variety of similar target genes, they seem to act in a non-redundant manner.[14] HIF-2α is the isoform mediating EPO synthesis of the cortical interstitial fibroblasts,[15,16] the major sources of EPO production in the kidney.[17] In fact, most HIF-based therapies for anaemic disorders (*vide infra*) mainly aim at HIF-2α stabilization.[18] On the contrary, HIF-1α seems to be the dominant isoform in the local response to ischaemic and hypoxic insults like stroke or heart attack, mainly regulating angiogenic factors (e.g. vascular endothelial growth factor, VEGF) and glycolytic enzymes.[19,20]

HIF hydroxylases

In 2001, a group of enzymes was discovered to be responsible for the specific hydroxylation of HIFα proline residues regulating its stability.[21–24] The so-called prolyl hydroxylase domain-containing proteins (PHD) belong to the class of non-haem iron-dependent dioxygenases that use O_2 and 2-oxoglutarate-(2-OG) as co-substrates for the hydroxylation of proline. Since their activity was found to be directly oxygen-controlled, they have been proposed as molecular O_2 sensors of the mammalian cell. The catalytic cycle of HIF-PHD enzymes begins with the coordination of Fe(II) and 2-OG to the active site, followed by sequential binding of the HIFα substrate and replacement of a water molecule by O_2. Subsequent decarboxylation of 2-OG produces a highly reactive Fe(IV) ferryl species that is responsible for the final HIF-prolyl hydroxylation, leading to the release of the citric acid cycle intermediate succinate and CO_2. Furthermore, ascorbate is required for full catalytic activity (for review see Nagel *et al.*[7] and Chowdhury *et al.*[25]).

The three different PHD isoforms (PHD1, PHD2, and PHD3, also referred to as EGLN2, EGLN1, and EGLN3, respectively) show an overlapping but distinct pattern of tissue distribution and have different specificity for the three HIFα isoforms.[26] PHD2 is the best studied HIF prolyl hydroxylase and is believed to play a critical role in the oxygen-dependent regulation of general HIFα stability.[16] It predominantly hydroxylates HIF-1α in normoxia but HIF-isoform selectivity declines with increasing enzyme activity. Hypoxia-induced PHD3 is reported to be more selective for HIF-2α, while PHD1 is not induced under hypoxic conditions and seems to play a more subtle role in the maintenance of oxygen homeostasis. The differential roles of HIF-PHDs in the pathogenesis of different diseases and the salutary response to such conditions are complicated, overlapping and controversies yet remain to be elucidated. Some additional information is given in the course of this work but for a detailed review the interested reader is referred to the literature.[7,10,18]

Besides the critical role of PHD oxygenases in the regulation of HIF stability, hydroxylation of a distinct asparagine residue of HIFα hampers the interaction between HIF and the co-activator complex p300/CBP. Since this is required for induction of gene transcription, asparagine hydroxylation inhibits HIF activity rather than decreasing its stability. Consequently, the enzyme catalysing the asparagine modification is termed Factor Inhibiting HIF (FIH).[27,28] Most therapeutic approaches aiming at the HIF-mediated stimulation of erythropoiesis, however, are based on inhibition of PHDs rather than FIH. This will be subject of the following sections.

HIF stabilization by prototypical PHD inhibitors (HIF-PHI)

The catalytic cycle of HIF prolyl hydroxylation offers several options of xenobiotic manipulation. Besides 2-OG and ascorbate, Fe(II) is a necessary co-factor for PHD activity and is reversibly bound to the active centre of the metalloenzyme. Hence, reduction of iron availability by competitive substitution with other catalytically incompetent metal ions (Co^{2+}, Ni^{2+}) or by iron-chelation, results in the inhibition of the enzyme activity.[21] In fact, administration of cobaltous (II) chloride is known to induce erythropoiesis and has been used in a clinical setting for the treatment of anaemia in the 1950s,[29] long before the HIF-PHD axis was unravelled. The erythropoietic stimulus of cobalt is potent enough to even produce polycythemia in humans[30] and, originally, the activity of an international EPO unit (IU) was referenced against the biological effect of 5 μM of cobaltous chloride.[31] Iron displacement in the catalytically active site of the PHD oxygenases is expected to be the dominant mechanism of cobalt-induced HIF stabilization[21] but interference with the ascorbate biosynthesis[32] and direct binding of cobalt to HIFα[33] have been proposed as alternative and/or additive ways of action. Adversely, soluble cobalt (II) salts are classified as group 2B carcinogens (possibly carcinogenic to humans) by the International Agency for Research on Cancer[34] and due to its toxic side effects their use as hypoxia-mimetic agent is mainly limited to experimental applications today.

One of the first organic compounds that was specifically shown to induce HIF activity was discovered in 1993 by Wang and Semenza, the same group that purified and characterized the HIF protein two years later.[9,35] The chelating agent desferrioxamine (DFO, also known as deferoxamine, Figure 1,

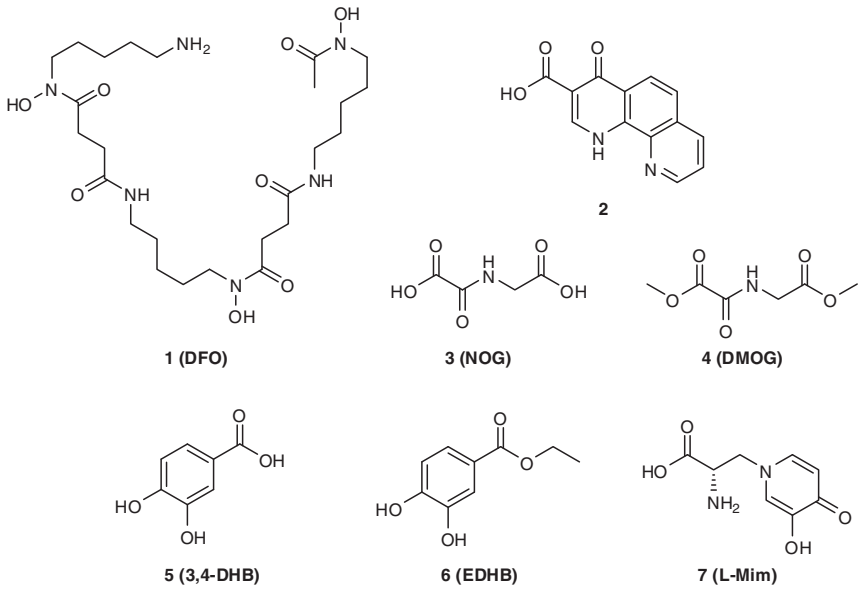


Figure 1. Molecular structures of prototypical HIF-PHI of the first generation.

compound **1**), an antidote for the clinical treatment of iron overloads, was confirmed to stabilize HIF and to up-regulate EPO RNA levels. However, it took several years to fully understand the mechanism underlying the HIF-stabilizing effect of this compound and, in fact, it played an important role in ultimately identifying the novel class of PHD enzymes that regulate HIF-stability by specific proline hydroxylation.[22,24] As described earlier, these findings marked the breakthrough in the search for the molecular mechanism of mammalian oxygen sensing.

In the same context, a variety of small molecule inhibitors of the related class of collagen prolyl hydroxylases (CPH), developed for the treatment of fibrotic diseases, were tested for their inhibitory effect on HIF-PHD. Low micromolar concentrations of the iron chelating 1,10-phenantroline derivative **2**, for example, were reported to decrease PHD activity, leading to an accumulation of HIF-1α in several cell culture models.[36]

Besides iron chelators, among the first CPH inhibitors that were intentionally used for the inhibition of HIF-PHD were 2-oxoglutarate mimetics, compounds that competitively bind to the active site of PHD but are not decarboxylated like the naturally bound co-substrate. *N*-oxalylglycine (NOG, Figure 1, compound **3**) and its cell permeable diester dimethyl-*N*-oxalylglycine (DMOG, compound **4**) are first-generation examples of 2-OG analogues that were shown to inhibit HIF-PHD activity but they seem to be neither potent nor selective enough to gain relevance in a clinical setting.[22,36] The glycineamide motif of these compounds, however, constitutes the structural basis for the majority of next-generation HIF-PHI.

3,4-dihydroxybenzoic acid (3,4-DHB, also known as protocatechuic acid, compound **5**) has been shown to stabilize HIFα *in vitro* and *in vivo* by replacing 2-OG and ascorbate[37] rather than iron as previously proposed.[38] Noteworthy, its esterified analogue ethyl-3,4-dihydroxybenzoate (EDHB, compound **6**, sometimes also referred to as 3,4-DHB) has been shown to raise serum EPO levels and to significantly increase exercise performance of mice under hypoxic conditions.[39] Administration of the natural, rare amino acid L-mimosine (compound **7**) to rats led to HIFα accumulation in the kidney and expression of HIF target genes in human cells.[38]

In compound **8** (S956711), which also emerged from the development of CPH inhibitors, the 1-carboxy function of NOG was replaced by a heterocyclic 2-quinolinyl substituent. It was reported to inhibit all three PHD isoforms[40] and to stabilize HIFα in the rat kidney.[38]

In 2006, two independent working groups succeeded in co-crystallizing a catalytically active domain of human PHD2 with the structurally related isoquinoline glycineamides **9** and **10** (this structure will be discussed later), respectively, and analyzed the purified complexes by X-ray crystallography.[41,42] The solved structures provided a new foundation for the understanding of the molecular mechanism of PHD catalysis and inhibition by 2-OG mimetics and offered new perspectives for the development of novel, more specific PHD inhibitors.

Compound **11** (PHD2 $IC_{50} = 4.2\,\mu M$) that emerged from one of these studies, is an example of a series of 8-hydroxyquinolines with variable substitution at the amide nitrogen and devoid of the glycineamide motif of the first generation HIF stabilizer.[43] The same group also identified a range of pyrazolopyridines and imidazo[1,2-a]pyridines as novel PHD inhibitors (not shown).[44–46]

Also based on the reported PHD2 crystal structure, two uracil-based HIF-PHI were designed by docking simulation studies and their potential to induce HIF-dependent genes and protect organs from ischaemic damage was tested.[47] TM6008 (compound **12**) was reported to inhibit PHD activity *in vitro* by 100% at a concentration of 20 μM. TM6089 (compound **13**) was found to be less active but, interestingly, when bound to the enzyme's active site, it accomplished its inhibitory effect without chelation of the Fe(II) atom and was therefore claimed to be the first PHD inhibitor without iron-chelating motif. This may be beneficial for the reduction of unwanted side-effects that could potentially occur due to non-specific iron binding during HIF-PHI therapy.

In recent years, researchers investigating the differential roles of the three PHD isoforms in different tissue could announce

some success, although the whole complexity of the signalling system is still not fully deciphered. For example, double PHD1/PHD3 deficiency was reported to activate the hepatic HIF-2α/EPO pathway while PHD2 gene disruption or inhibition seems to induce erythropoiesis via the renal pathway.[48] Given the host of targets genes regulated by HIF in different organs (EPO, VEGF, HO-1, GLUT, amongst hundreds of others) a selective, even tissue-specific inhibition of the PHD isoforms would be a desirable tool for the development of specific therapies for certain diseases. A study with mice showed that inactivation of PHD1 improved the hypoxia tolerance of skeletal muscle by switching the glucose metabolism from oxidative to anaerobic ATP production.[49] Interestingly, this led to decreased exercise capacity under normoxic conditions, indicating that PHD inhibition could also trigger physiological responses that are detrimental to aerobic endurance performance. Aragones *et al.*[49] achieved disruption of PHD1 exclusively by approaches of genetic manipulation, because no small-molecule inhibitor specific for PHD1 was available.

The first HIF-PHI that possess considerable selectivity for different PHD isoforms were recently reported by Murray *et al.*[50] Their approach was based on a previously patented HIF-PHI with quinolone glycineamide core[51] that the authors modified with different dipeptidyl-substitutents in the 6-position of the heterocycle. Compound **14**, the most selective molecule of this series, was found to be 30–40-fold more potent against PHD1 and PHD3 than against PHD2. These results might yield new perspectives for the structure based design of novel, even more specific inhibitors with the potential to give rise to yet another generation of HIF-PHIs.

Recently, some aspirin metabolites were identified to be inhibitors of HIF-PHD2.[52] The dihydroxybenzoylglycine **15**, which was found in human urine after aspirin intake, had a PHD2 IC_{50} value of 27.4 μM in an enzyme based assay and its cell permeable ethyl ester derivative was shown to stabilize HIF-1α in various human cell lines even more potently than did DMOG. If HIF-stabilisation by aspirin metabolites is also observed *in vivo* and potentially contributes to the prophylactic pharmacologic effects of the widely used drug, yet remains to be investigated. Studies assessing the influence of aspirin on athletes' performance mainly concentrate on the anti-inflammatory and analgesic effects of the drug.[53] The same applies for discussions about the widespread use of aspirin and related nonsteroidal anti-inflammatory drugs (NSAID) amongst recreational and professional athletes.[54]

Patent claims of compounds for HIF-PHI therapy

Fibrogen Inc. (San Francisco, CA, USA) was early devoted to the investigation of collagen prolyl hydroxylases (CPH) and their inhibition for therapeutic purposes. When the pivotal role of PHD enzymes as oxygen sensors was discovered in 2001, Fibrogen's extensive library of small-molecule CPH inhibitors assured the company a leading position in the development of novel inhibitors for HIF-specific prolyl hydroxylases. Compound **10**, which is proposed to correspond to the structure of FG-2216, Fibrogen's first lead drug candidate for the treatment of CKD-related anaemia, was initially described together with a series of substituted isoquinoline-3-carboxamides as inhibitor of CPH in a patent from 1998.[55] The reported CPH IC_{50} of **10** was 2.3 μM, while example **16** was found to be the most potent CPH inhibitor of this set of compounds with an IC_{50} for CPH of 0.12 μM. The first patents describing small molecules for the stabilisation of HIFα by PHD inhibition were filed in 2003, disclosing several core structures, including substituted carboxamides of sulfonamides, 1,10-phenantrolines, cinnolines, pyridines, pyridazines, quinolines and isoquinolines.[56,57] In these patents, the erythropoiesis-stimulating effects were most comprehensively discussed for compound **10** but also oral administration of **16** (100 mg/kg/day for 3 days) was shown to clearly increase plasma EPO and haematocrit (Ht) levels in mice after two days. Subsequently, the ability of the isoquinoline glycineamide **10** to act as HIF-PHI, to increase serum EPO levels in healthy humans and to stimulate the Hb response in anaemic CKD patients has been described by Fibrogen in patents from 2004 and 2006.[58,59] Although **10** was again the most prominently described example and the only substance administered to human subjects, repeated oral doses of 20 mg/kg of compound **17** was reported to be the most potent enhancer of Hb formation in mice. In a recent publication, it was shown that preconditional HIF-PHD inhibition with compound **10** in a model of renal ischemia was more effective in the protection of the renal function than treatment with rhEPO.[60]

In the course of further structure optimisation, Fibrogen discovered that incorporation of a cyano-substituent at the C1 position of the isoquinoline heterocycle strongly improved the EPO-inducing features of the resulting molecules.[61] Compounds **18** and **19** have 4.7- and 449-fold increased effects on EPO production, respectively, when compared to their analogues with chloro substituent in C1 position. Interestingly and alarming for anti-doping authorities, a selection of substituted C1-cyano, -chloro and -methyl isoquinoline-3-carboxamides, including **18**, **19**, and other compounds that have been reported as HIF-PH inhibitors, seem to be commercially available.

General structure **II** describes a great variety of Fibrogen's novel glycineamide PHD inhibitors with fused pyrrolo-, thiazolo-, isoxazolo- and oxazolo-pyridine heterocycles, published between 2007 and 2009.[62–64] Noteworthy, the [(3-hydroxypyridine-2-carbonyl)-amino]-acetic acid core still resembles the isoquinoline glycineamide motif of the first generation HIF-PHI.

The same pyridine-based pharmacophore was chosen by Proctor & Gamble Pharmaceuticals (P&G, Mason, OH, USA) for the design of HIF stabilizing PHD inhibitors in a 2007 patent application.[65] The structure and activity of compound **20** was disclosed together with numerous other pyridine glycineamides with a variably substituted phenyl residue linked to the pyridine-C5 atom. Compound **20** was reported to be the most potent inhibitor of PHD2 (IC_{50} = 99 nM), also effecting an EPO response in mice upon oral dosing. Other analogues with high inhibitory effects on PHD2 activity and the capacity of EPO induction are listed in the patent publication and include the 4-chlorophenyl- (PHD2 IC_{50} = 240 nM), 3-chlorophenyl (IC_{50} = 1100 nM) and the 3-cyanophenyl-substituted (IC_{50} = 190 nM, EPO response not determined) analogues, respectively (not shown).

Crystal Genomics, Inc. (Seoul, Korea) published a patent in 2009, also claiming the use of numerous pyridine carboxamides as pharmaceutical preparations for the modulation of HIF and the treatment of related disorders (not shown).[66]

A novel pharmacophore of the following generations of HIF-PHI, explored by several pharmaceutical companies, comprises a [[(4-hydroxy-2-pyridon-3-yl)carbonyl]amino]acetic acid scaffold with variable carbon or heteroatoms (mainly nitrogen) in the positions 5 and 6 of the heterocycle and often fused to 5- or 6-membered saturated or aromatic (hetero-) cycles (general structure **III**). GlaxoSmithKline, Inc. (GSK, Philadelphia, PA,

USA), besides Fibrogen another key player in the research field of HIF-mediated therapies of various disorders, was one of the first companies to follow this approach for the development of PHD inhibitors.

A series of 4-hydroxy-2-quinolinone glycineamides was among the first HIF-PHI disclosed by GSK in 2007 and claimed for the treatment of anaemia, having PHD3 IC_{50} values of 20–1000 nM and EPO EC_{50} between 1 and 20 μM.[67] Compounds **21** and **22** were the two examples of the patent that were additionally prepared in large scale (89 g). The molecular structure of **21** was recently attributed to the investigational HIF-PHI GSK360A in a GSK-research paper published by Bao *et al*., reporting the capability of HIF-PHI therapy to protect the heart after myocardial infarction in a rat model.[68] In an enzymatic assay, compound **21** reversibly inhibited the three different PHD isoforms with IC_{50} values of 10, 100, and 126 nM for PHD1, PHD2, and PHD3, respectively. The EPO and VEGF EC_{50} were determined to be at 3.2 and 1.3 μM, respectively, and a 13-fold increase of EPO production was found in Hep3B cells at 3 μM substrate concentration. A single oral dose of 30 mg/kg administered to rats, resulted in a maximum plasma drug concentration (C_{max}) of 27162 ng/ml after 4 h and decreased to half C_{max} ($T_{1/2}$) after 6.5 h. After chronic oral treatment with GSK360A (30 mg/kg/day) once daily for 4 weeks, a robust increase of the haematologic parameters RBC, Hb and Ht was observed.

Another representative of GSK's first prolyl hydroxylase inhibitors is the series of *N*-substituted pyrimidinetrione glycineamides, exemplified by compound **23** and also disclosed in a 2007 patent.[69] PHD3 IC_{50} values ranged from 0.8 nM to 20 μM and EPO EC_{50} was between 0.4 and 100 μM (no inhibitory data were presented for specific examples). Example **23** was the only compound additionally synthesized in a large-scale method (116 g).

The same core motif **III** was applied by Amgen, Inc. (Thousand Oaks, CA, USA) for the development of numerous quinolones, azaquinolones and diazaquinolones that were patented for the use as HIF-PH inhibitors in 2007 and 2008 (not shown).[51,70,71] A new approach was published by Amgen in a 2008 patent, taking the same quinolone core structure as described above but modifying the amide position of the glycineamide side chain.[72] Compound **24**, comprising a methylene unit instead of the amide nitrogen, had a more than 80-fold increased PHD2-selectivity against CPH1 (compared to the corresponding glycineamide analogue) and elevated EPO levels by a factor of approximately 25, when given orally to rats (50 mg/kg).

Numerous strategies for the development of non-glycineamide based HIF-PHI were followed by GSK, Merck, Amgen, P&G, Crystal Genomics and Johnson & Johnson (numerous exemplary structures are listed in the works by Yan *et al*.[73] and Rabinowitz *et al*.[74]) but only the set of compounds from Bayer Healthcare (Leverkusen, Germany), which is devoid of the common pharmacophores, shall be presented here. The conserved core motif of most of Bayer's HIF-PHD inhibitors is a dihydropyrazolone scaffold with different 5- or 6-membererd heterocycles like pyridine, pyrimidine, imidazole or triazole as substituents in the 2- and 4-position, respectively.[75–79] Compound **25** is a representative example of Bayer's dihydropyrazolone-based HIF-stabilizers and reported as the most potent inhibitor of PHD activity (no isoform specification) with an IC_{50} value of 50 nM.[78] Moreover, all presented substances were claimed to elevate plasma EPO as well as Ret, RBC and Ht levels in mice and rats. Moreover, in all patents Bayer provided detailed information about LC-MS detection methods and specified the analytical parameters retention time (RT) and *m/z* values for each of the disclosed molecules.

HIF-PHI therapies in clinical trials

Being one of the pioneers in the field of commercial research on prolyl hydroxylase inhibition and related patent claims, Fibrogen also runs clinical trials for the therapeutic use of HIF stabilizers in the most advanced stages. FG-2216, the lead drug candidate for the treatment of CKD-related anaemia, has completed Phase II trials, while the second generation HIF-PHI FG-4592 is currently enrolling for Phase IIa and IIb trials in the USA.[80] Fibrogen has licensed the development of both drugs in other parts of the world including Japan and Europe to Astellas Pharma, Inc. (Tokyo, Japan), where they are listed under the code names YM 311 and ASP-1517, respectively. To date, Fibrogen claims to have conducted clinical studies with HIF-PHIs involving nearly 700 patients. In 2004, FG-2216 was reported to significantly raise serum EPO levels in healthy human subjects receiving doses between 6 and 20 mg/kg drug given 3 times weekly for 3 weeks.[81] This Phase I clinical trial showed, for the first time, that orally available small molecule HIF-PHIs are capable of up-regulating the endogenous EPO production in humans, thus affecting the natural erythropoietic system. Moreover, Fibrogen's HIF-PHIs are claimed to stimulate the complete erythropoietic machinery including red blood cell maturation and iron mobilisation, because a quick and significant rise in Hb levels was observed even in those cases, where plasma EPO levels were only modestly increased.[82] This might be advantageous in the course of HIF-PHI therapy, because supra-physiologic levels of circulating EPO that are observed in the case of conventional rhEPO administration, are associated with an increased risk of erythrocytosis and other adverse side effects. However, if the safety profile of long-term HIF prolyl hydroxylase inhibition is superior to rhEPO therapy, yet remains to be clarified. Numerous concerns about potential unspecific effects of manipulating the PHD-HIF axis, e.g. the role of HIF in pathologic processes like tumour growth, pulmonary hypertension or diabetic retinopathies,[7] will have to be addressed.

Although not officially confirmed yet, the molecular structure of FG-2216 appears to correspond to compound **10** (Figures 2 and 3).[73,83] In the first peer-reviewed report of FG-2216 efficacy, oral administration of the drug to rhesus macaques for several weeks significantly increased EPO and Hb levels by stabilisation of HIF-2α.[84] However, the only peer-reviewed article showing the EPO-inducing effect of FG-2216 in humans was just recently published by Bernhardt *et al*.[85] In the study including also nephric and anephric CKD patients, healthy human subjects receiving a single oral dose of 1250 mg of FG-2216 (20 mg/kg) experienced a 12.7-fold mean increase of plasma EPO levels 12 h after the administration. Additionally, pharmacokinetic data were provided that will be discussed later (*vide infra*). In 2007, the clinical trials of Fibrogen's HIF-PHIs were temporarily paused after a case of death due to fulminant hepatitis occurred in the course of a phase II study. Although the adverse event was declared not to be drug-related and the FDA permitted to fully resume the tests, no clinical trials with FG-2216 involving human subjects have been conducted/reported since.

FG-4592, Fibrogen's back-up candidate for the treatment of CKD-related anaemia, is described as a second-generation HIF-PHI with improved pharmacokinetic and pharmacodynamic

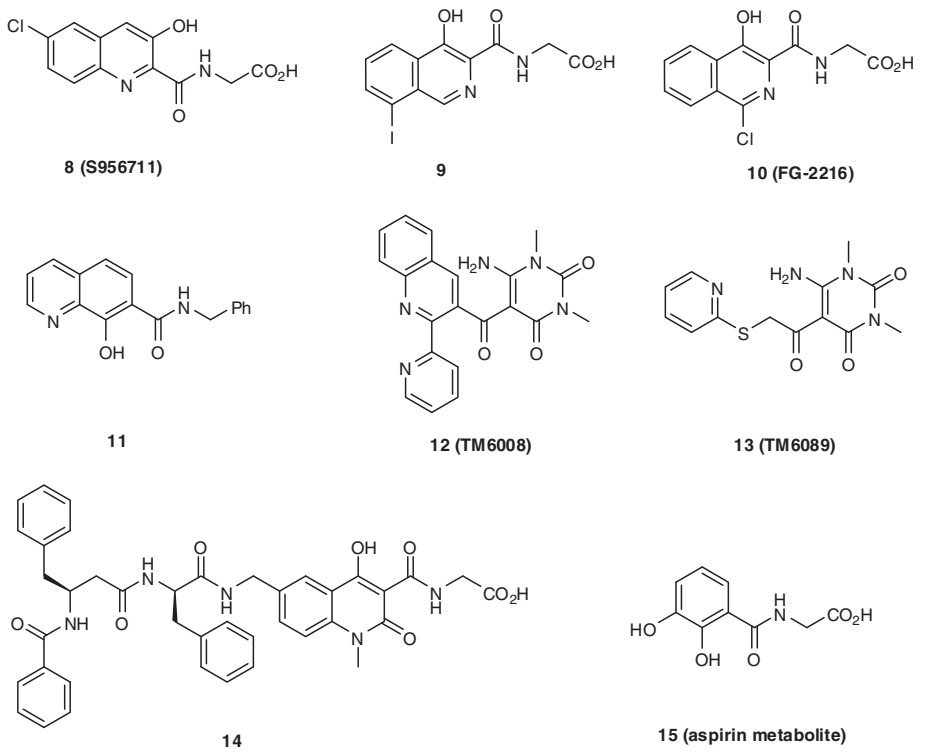


Figure 2. Molecular structures of prototypical HIF-PHI and investigational next-generation HIF-PHI.

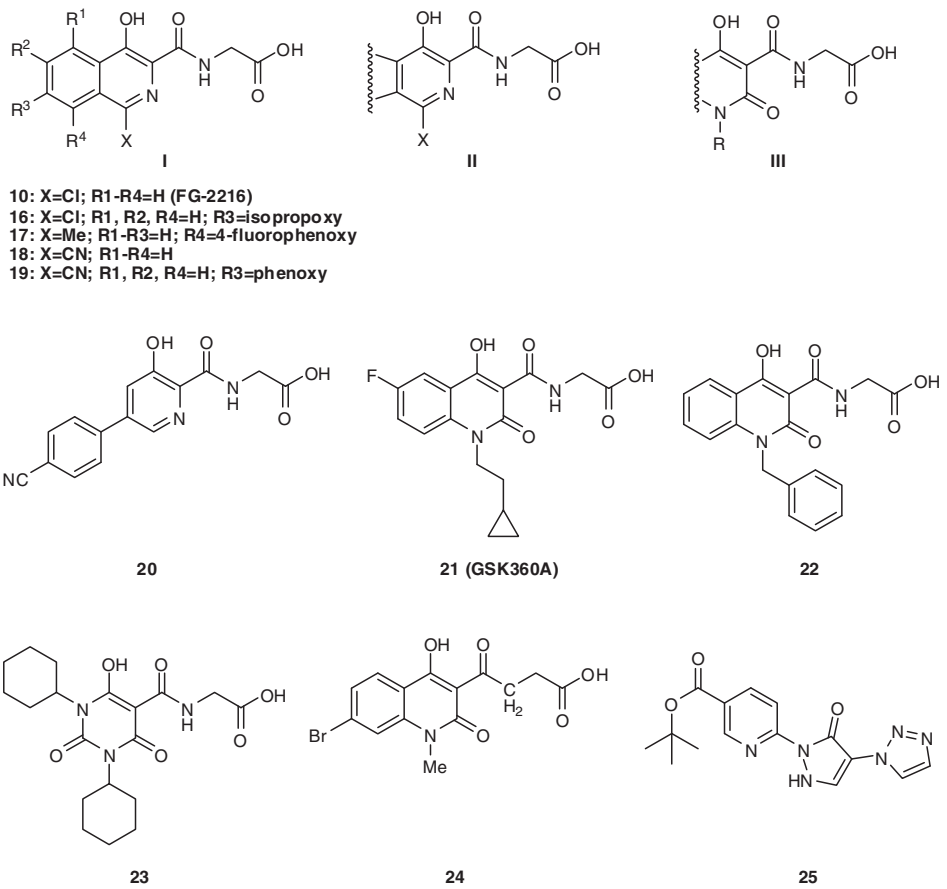


Figure 3. General structures **I-III**, summarizing the majority of patented HIF-PHI molecules and exemplary compounds **16–25**.

parameters and was first mentioned to stabilize HIF-2α *in vitro* and *in vivo* in 2005.[86] More recently, in the course of a Phase II clinical study with anaemic CKD patients, oral treatment with FG-4592 three times per week with doses ranging from 0.7 to 2 mg/kg resulted in a mean increase of Hb between 1.0 ± 0.9 and 2.2 ± 0.8 g/dl, respectively.[87] The administered doses are approximately 10-fold lower than those given in the FG-2216 studies, indicating a higher potency of FG-4592 to inhibit HIF prolyl hydroxylases and to induce erythropoiesis. Due to the quick successive chronology of the first references, it seems likely that FG-4592 was selected from the same compound set as FG-2216 or arose from optimisation of the FG-2216 lead structure. It is therefore conceivable that it is structurally related to its predecessor, although the exact molecular structure of FG-4592 is still undisclosed.

Moreover, Fibrogen owns an extensive library of experimental HIF-PHIs that have not (yet) been tested in a clinical setting but whose efficacy has been shown in the investigation of different disease models. Examples of such substances are FG-4497,[15,88,89] FG-4487,[90] and FG-4383.[91]

GlaxoSmithKline is testing its investigational HIF prolyl hydroxylase inhibitor GSK1278863 for the treatment of anaemia since 2008 and has completed several Phase I studies with healthy human subjects in the USA, testing parameters like bioavailability, pharmacokinetics and pharmacodynamics, safety and tolerability of single and repeated doses ranging between 2 and 450 mg.[92–95] Two Phase I studies with renally impaired subjects are scheduled in New Zealand and the USA.[96,97] Currently, Phase II studies are running in Australia, New Zealand, India, and the Russian Federation with anaemic pre-dialysis and hemodialysis-dependent patients repeatedly receiving doses between 25 and 100 mg for 28 days.[98] Moreover, GSK1278863 is not only tested for the treatment of CKD-related anaemia but is also reported to be in Phase II clinical trials for the treatment of peripheral arterial disease according to GSK's 2011 product pipeline.[99]

Recently, the drug candidate GSK360A (Figure 3, compound **21**) entered the stage of GSK's pre-clinical development program. Oral administration of the HIF-PH inhibitor was reported to mediate cardioprotective effects and to raise EPO and Hb levels in rats.[68] Although, first mentioned already in 2008, no information about the progress of development is available from GlaxoSmithKline and no data or plans of administration studies with human subjects have been published.

Akebia Therapeutics, Inc. (Cincinnati, OH, USA) was founded in 2007, in-licensing pre-clinical programmes on HIF-PHIs from Procter & Gamble. Its lead drug candidate AKB-6548 (structure undisclosed) is an orally bioavailable, predominantly HIF-2α stabilising prolyl hydroxylase inhibitor for the treatment of chronic anaemia that has completed Phase I clinical trials with 48 healthy volunteers.[100] In a Phase IIa study with 22 CKD patients, EPO levels were elevated 8 and 12 h after a single oral dosage of AKB-6548 (no dose reported) and returned to baseline values 24 h after the administration.[101] Akebia is currently recruiting anaemic CKD patients for a Phase II trial, testing the change in haemoglobin levels during once-daily administration of different doses of the drug (240, 370, 500, and 630 mg) for 42 consecutive days. The facts that, according to our literature research, P&G has filed only one patent claiming small-molecule PHD inhibitors for therapeutic use and the first patents from Akebia in this field are from 2011, narrow the group of potential candidates for the molecular structure of AKB-6548 to the pyridine-based compounds disclosed in the 2007 patent discussed earlier.[65]

AKB-4924 is another HIF-PHI currently being tested by Akebia in preclinical studies. This compound, however, is reported to predominantly stabilize HIF-1α and is designed for the support of wound healing and the treatment of irritable bowel disease,[102] thus possessing less potential for the misuse as performance enhancing agent in sports.

The investigational HIF stabilizer BAY85-3934 from Bayer Healthcare has shown a promising profile in preclinical studies and two Phase I clinical trials are currently recruiting participants (healthy volunteers and CKD patients with renal anaemia) in Germany and the United Kingdom. In one study, 80 participants are to receive a single oral dose of 20 mg (optional 40 mg) of BAY85-3934,[103] while the second trial is a combined single/multiple dose escalation study with 48 CKD patients receiving up to 650 mg of drug in 13 days.[104]

In 2008, the drug discovery and development company Crystal Genomics has started a strategic alliance with the US firm ProQuest Investments and out-licenced the clinical development of their novel HIF-PH inhibitors to the newly founded company Palkion, Inc. According to communications on the company's website (www.cgpharma.com), the lead drug compound has shown good efficacy in monkeys and has reached the last stages of pre-clinical testing. No further information is publicly available at the moment.

Although Merck & Co., Inc. (Rahway, NJ, USA) has filed a series of patent applications claiming the use of HIF-PH inhibitors for the treatment of anaemic and ischaemic diseases, no programs for the clinical development of novel HIF stabilising agents are reported. The same fact applies to Amgen, Inc. and Johnson & Johnson, Inc. (New Brunswick, NJ, USA) even though both companies are among the world's leading sellers of protein-based erythropoiesis stimulating agents.

Doping relevance of HIF-PHI and their detection in sports drug testing

As outlined in the previous sections, the possibilities to artificially enhance human erythropoiesis by small-molecule xenobiotics are manifold and rapidly expanding, due to the ongoing progress in understanding the cellular and molecular responses to hypoxia. WADA has responded to the potential threat that emanates from the increasing number of emerging HIF-PHIs by explicitly banning the use of HIF stabilizers (2011 Prohibited List, category S2.1) as well as any other non-approved substance (category S0) in sports.[2]

Various investigation agents are now routinely used to mimic the effects of oxygen deprivation in experimental laboratory settings and their efficacy is often documented in cell culture or animal models (the relevancy of this group of prototypical substances is discussed later in this chapter). To date, however, no HIF-PHI is available that has gained clinical approval for the use as erythropoiesis-stimulating agent in humans. This makes emerging drugs with demonstrated safety and tolerability hard to obtain by athletes with the intention of misusing them as performance enhancing agents in sports. This unavailability simultaneously poses a profound challenge for doping analysts: a targeted screening for these substances implicitly relies on structural information about the active drug but this data is often kept under strict disclosure by the pharmaceutical companies in the time of clinical trials. It is therefore an important task of preventive doping research to gain

information about emerging drug candidates in the earliest possible stage of clinical investigation or, if this is not possible, to find adequate model compounds that resemble the core structures of the supposed active drug and support the development of analytical assays for sports drug testing.

Targeted detection assays

The best and first option to test for novel doping agents like HIF-PHI is usually a targeted screening for the known substances, due to superb sensitivity and specificity of the relevant methods. To the best of our knowledge, Fibrogen is the only company that has disclosed the molecular structure of a HIF-PHI, whose erythropoietic stimulus has been tested and verified in human subjects (compound **10**).[59,60] Although not officially confirmed yet, this molecule seems to correspond to FG-2216. As was recently published by Bernhardt *et al*., oral administration of FG-2216 to healthy humans resulted in the excretion of a considerable amount of the unchanged drug compound into urine within 48 h after administration.[85] Therefore, the active drug itself can serve as a urinary biomarker for the abuse of FG-2216 and other structure related HIF-PHI, potentially like FG-4592. Nonetheless, information about the metabolic fate of these substances is of great importance for sports drug testing, because metabolites can potentially be excreted into the urine for a longer time span than the respective parent compound.[105] Consequently, screening for such analytes would result in an extended detection period. We recently reported the first analytical liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) methods for the determination of HIF-stabilizing isoquinoline-3-carboxamides, including compounds **10** (FG-2216) and **16**, from human urine, the predominant biological matrix used in routine doping control.[106] Analysis of the samples was achieved by direct injection of urine into the LC-MS system and detection of target compounds by electrospray ionisation in positive and negative ion mode, followed by diagnostic multiple reaction monitoring (MRM). In negative ESI mode, the method was fully validated, with lower limits of detection (LLOQ) of 10 and 2 ng/ml for compounds **10** and **16**, respectively. We also conducted *in vitro* metabolism studies with **10** and model HIF stabilizer **16**, in order to expand the list of potential urinary target analytes for this class of compounds. The provided mass spectral and chromatographic data enable the implementation of these analytes into the routine screening procedures of other doping control laboratories. For the official report of an adverse analytical finding fulfilling WADA guidelines, however, the direct comparison of the suspicious urine specimen to reference material is required. The distribution of such standards to all accredited doping laboratories by WADA, which might have access to drugs in early development by cooperation agreements with pharmaceutical companies, would facilitate the effective screening for this class of emerging drug candidates. Additionally, a more comprehensive LC-MS/MS method was developed based on the mass spectrometric characteristics that were identified in previous works with protonated isoquinoline carboxamides.[83,107] Multiple, spontaneous ion-molecule reactions between specific product ions and residual water in the gas phase of the MS result in unusual nominal losses of 10 and 11 Da in the course of the dissociation pathway. An LC-ESI-MS/MS neutral loss scan for 10 Da was subsequently shown to be capable of detecting isoquinoline-3-carboxamides from human urine, irrespective of their substitution pattern. The comprehensive screening approach also detects substances with unknown related structure and can potentially be expanded to tackle the current challenges of preventive doping research, e.g. detecting the misuse of yet undisclosed HIF-stabilising ESA (*vide infra*).

Strikingly, Bayer's announcement to recruit healthy human subjects for the first Phase I clinical trial of its drug candidate BAY85-3934, has recently been subject of discussions on cyclists' Internet platforms. Although such findings should not be overrated, they clearly show that the current progress in clinical HIF-PHI therapy is not exclusively reserved to therapeutically oriented target groups. As described earlier, Bayer's lead compound was probably recruited from one of the disclosed sets of dihydropyrazolones that are all devoid of a glycineamide motif. Most of the specified compounds are of predominantly basic nature, proposing that positive ionisation will yield the best analytical results here. A preliminary LC-ESI-MS screening method could be developed based on the data provided in Bayer's patents for each of the compounds, including a detailed description of the instrumental setup.[76–79] However, the given parameters retention time and *m/z* value of the protonated molecules are not sufficient to fulfil WADA requirements of validated analytical procedures and more data on the MS/MS features of Bayer's HIF-stabilizers are indispensable for the development of routine doping control methods.

GlaxoSmithKline's novel drug candidate GSK360A (compound **21**)[68] has recently attracted the spotlight of anti-doping research. In the abovementioned publication by Bao *et al*., chronic HIF-PHI therapy with GSK360A had numerous beneficial effects on the cardiac function and morphology after induced myocardial infarction but it also led to a significant increase in expression of the HIF target genes haemeoxygenase-1 (HO-1), pyruvate dehydrogenase kinase isozyme 1 (PDK1) and transferrin in the skeletal muscle. Although the need of further studying the role of skeletal muscles in this context is emphasized, the authors strikingly conclude that 'PHD inhibition can enhance exercise performance and convey hypoxic tolerance'. With this concise statement, the data have the potential to be recognized by communities that are not primarily interested in a therapeutic application of HIF-stabilizers, once again underlining the high relevance of this novel compound class for anti-doping approaches. Noteworthy however, contradictory effects of inhibition or germline disruption of specific PHD isoforms on exercise performance have been published and discussed in the literature (*vide supra*).[7,49]

A detection assay for the novel drug candidate has not yet been reported but the disclosed structure enables a timely implementation of this analyte into routine LC-MS/MS doping control procedures, presuming synthesis and mass spectral characterisation of reference material is conducted.

The drug candidate GSK1278863 (structure undisclosed) is converted into six metabolites, three of which are circulating in the plasma at concentrations greater than 10% of the active drug, according to a clinical pharmacology protocol available by download from the GSK website.[108] Unfortunately, no further information is given in the document and analytical methods are not yet available.

Besides the targeted detection assays relying on the knowledge of the compounds' MS/MS-behaviour, modern high resolution/high accuracy MS techniques offer new possibilities for the determination of emerging drugs in doping analysis.[109]

Extracted ion chromatograms based on accurate masses of known target analytes might potentially yield a sufficient specificity to develop a preliminary LC- and/or GC-MS(/MS) screening method for HIF-PHIs. Confirmation of suspicious findings, however, would necessitate a reliable determination of the definite molecular structure corresponding to the accurate *m/z* value (*vide infra*). Table 1 summarizes the information about all small-molecule ESAs presented in this review, including molecular compositions and accurate masses for the support of further analytical assay development.

Other potential detection strategies

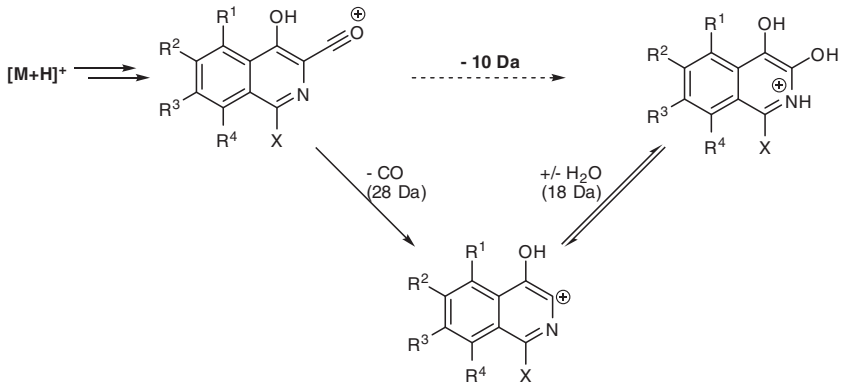
As discussed earlier, a targeted LC-MS screening is impossible, if detailed information about the emerging drugs' structure and its mass spectral characteristics are not yet available. In this case, alternative approaches, for example, aiming at the detection of repetitious structural motifs, have to be probed. Due to the plethora of HIF-PHI claimed for clinical use (a more comprehensive overview is given by Yan *et al.*[73] and Rabinowitz *et al.*[74]), it is advisable to summarize the numerous structures of individual compounds into several super-groups (e.g. general structures **I**, **II** and **III**, Figure 3). Having a closer look at these compound classes, several structural patterns occurring in the majority of molecules can be highlighted. The glycineamide chain that was adapted from the first-generation prolyl hydroxylase inhibitor *N*-oxalylglycine is the most conserved molecular motif. Apart from Bayer, which is following an entirely different structural approach, all pharmaceutical companies working on HIF-PHI therapy have claimed patents for compounds comprising this glycineamide scaffold. This will prove helpful for the development of non-targeted mass spectrometry based screening methods that should be able to detect whole compound classes, independent of the definite molecular structures of its individual members.

The previously presented approach based on a neutral loss scan for 10 Da (Scheme 1) detects a variety of glycineamides bound to isoquinolines and probably other aromatic heterocycles like quinolines or pyridines. This would potentially cover various patented HIF-PHI from Fibrogen, Amgen, Procter & Gamble and Chrystal Genomics. It is unlikely, however, that the same CID behaviour can be observed for the non-aromatic HIF-PHI with the pyridone-based pharmacophore (general structure **III**). This will have to be subject of future studies. Other drawbacks of the described method are the unsatisfactory sensitivity, with LODs ranging from 300 to 1000 ng/ml, and the need

Table 1. Prototypical and therapeutic small-molecule ESAs.

Cmp No	Name	Biol. target	Clinical stage	Accurate Mass	Elemental Composition	Proposed Detection
1	DFO	HIF	Prototype	560.3534	$C_{25}H_{48}N_6O_8$	LC-MS
2	FG-0041	HIF	Prototype	240.0535	$C_{13}H_8N_2O_3$	LC/GC-MS
3	NOG	HIF	Prototype	147.0168	$C_4H_5NO_5$	LC/GC-MS
4	DMOG	HIF	Prototype	175.0481	$C_6H_9NO_5$	LC/GC-MS
5	3,4-DHB	HIF	Prototype	154.0266	$C_7H_6O_4$	LC/GC-MS
6	EDHB	HIF	Prototype	182.0579	$C_9H_{10}O_4$	LC/GC-MS
7	L-Mim	HIF	Prototype	198.0641	$C_8H_{10}N_2O_4$	LC/GC-MS
8	S956711	HIF	Prototype	280.0251	$C_{12}H_9N_2O_4Cl$	LC/GC-MS
9	-	HIF	Prototype	371.9607	$C_{12}H_9N_2O_4I$	LC/GC-MS
10	FG-2216*	HIF	Phase II	280.0251	$C_{12}H_9ClN_2O_4$	LC/GC-MS°
11	-	HIF	Prototype	278.1055	$C_{17}H_{14}N_2O_5$	LC/GC-MS
12	TM6008	HIF	Prototype	387.1331	$C_{21}H_{17}N_5O_3$	LC-MS
13	TM6089	HIF	Prototype	306.0787	$C_{13}H_{14}N_4O_3S$	LC-MS
14	-	HIF	Prototype	717.2799	$C_{40}H_{39}N_5O_8$	LC-MS
15	Aspirin metabolite	HIF	Prototype	211.0481	$C_9H_9NO_5$	LC/GC-MS
16	FG-4592#	HIF	phase II	338.0669	$C_{15}H_{15}N_2O_5Cl$	LC/GC-MS
17	FG-4592#	HIF	phase II	370.0965	$C_{19}H_{15}N_2O_5F$	LC/GC-MS
18	FG-?	HIF	?	271.0593	$C_{13}H_9N_3O_4$	LC/GC-MS
19	FG-?	HIF	?	363.0855	$C_{19}H_{13}N_3O_5$	LC/GC-MS
20	AKB-6548#	HIF	phase II	297.0750	$C_{15}H_{11}N_3O_4$	LC/GC-MS
21	GSK360A	HIF	pre-clinical	348.1121	$C_{17}H_{17}N_2O_5F$	LC-MS
22	GSK1278863#	HIF	phase II	352.1059	$C_{19}H_{16}N_2O_5$	LC-MS
23	GSK1278863#	HIF	phase II	393.1900	$C_{19}H_{27}N_3O_6$	LC-MS
24	Amgen	HIF	?	352.9899	$C_{14}H_{12}NO_5Br$	LC/GC-MS
25	BAY85-3934#	HIF	phase I	328.1284	$C_{15}H_{16}N_6O_3$	LC-MS
26	K7174	GATA	pre-clinical	568.3512	$C_{33}H_{48}N_2O_6$	LC-MS
27	K-11706#	GATA	pre-clinical	720.3523	$C_{42}H_{48}N_4O_7$	LC-MS
28	PBI-1402#	BFU-E	phase II	144.1150	$C_8H_{16}N_2$	LC/GC-MS
29	PBI-1402#	BFU-E	phase II	470.3607	$C_{27}H_{50}O_6$	LC/GC-MS
30	PBI-1402#	BFU-E	phase II	554.4546	$C_{33}H_{62}O_6$	LC/GC-MS

* structure proposed;
# exemplary structure proposed



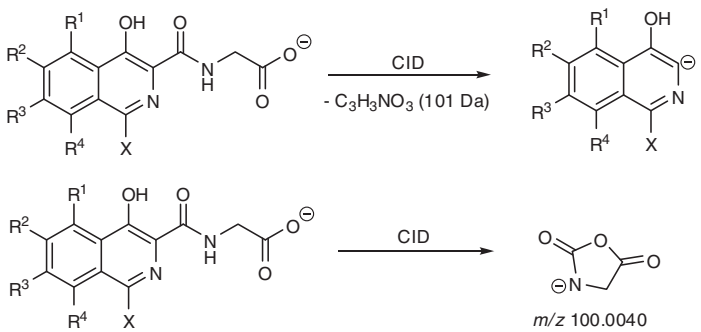
Scheme 1. The proposed general collision-induced dissociation pathway of isoquinoline-3-carboxamides includes a reversible ion-molecule reaction with gas phase water and an apparent neutral loss of a fragment of 10 Da.

for laborious confirmation methods to unambiguously assign a molecular structure to the compound displaying the detected unusual CID characteristics. In case of suspicious screening results, this might include different LC-MS^n techniques using high-resolution/high accuracy MS or even LC-NMR analysis for definite structure confirmation, as conducted in our recently published metabolism studies.[106]

A common CID reaction of deprotonated isoquinoline-3-glycineamides upon negative ESI was found to be the direct elimination of the carboxamide side chain (-$C_3H_3NO_3$, 101 Da, presumably eliminated as 2,5-oxazolidinedione, Scheme 2) from the respective precursor ion.[106] A similar dissociation reaction with the negative charge remaining on the side chain was also observed, leading to the product ion with *m/z* 100.0040 ($C_3H_2NO_3$, Scheme 2). This might offer two novel analytical approaches: both, a neutral loss scan for the leaving group of 101 Da as well as a precursor ion scan on *m/z* 100.0040 are reasonable approaches to detect a great variety of glycineamides bound to variety of (hetero)cyclic moieties. This approach might profit from the fact that, in most of the compounds, the terminal carboxylic acid group of the glycineamide is the only functionality with acidic properties. Therefore, deprotonation during ionisation and primary CID reactions probably occur at this site of the molecules. It is very likely that isoquinoline-, quinoline-, cinnoline- and pyridine-glycineamides would be covered by this potential analytical approach. Parameters like the specificity of this promising LC-MS/MS tool for the detection of WADA-prohibited ESA in urine samples, however, remain to be determined. High resolution MS (HRMS) approaches, for example, applying Orbitrap or Q-TOF detectors, are likely to yield the most specific analytical results, due to the additional analytical dimension provided by exact and accurate mass measurements. Moreover, it is yet to be investigated, whether the elimination of charged and/or neutral 2,5-oxazolidinedione also occurs upon CID of the non-aromatic compounds with a pyridone-based pharmacophore of general structure **III**, exemplifying a major part of newest generation HIF-PHI according to a review of according patents.[73] Model substances for this novel class of compounds have not yet been described in the literature.

Doping relevance of prototypical HIF-PHI

As presented and discussed earlier, numerous prototypical and experimental HIF-PHI that are not (yet) meant for clinical development of anti-anaemia therapies have been discovered in recent years. Strictly speaking, cobaltous chloride, as the first compound in this series, cannot be termed prototypical because it has effectively been used in humans for the therapeutic treatment of anaemia for years. The deleterious side effects and the consequent classification as carcinogen, however, completely removed it from the list of today's clinically relevant ESA therapies[110] and limit its use to experimental applications. Nevertheless, an intended misuse as doping agent cannot be excluded and has repeatedly been discussed in the past.[111–113] The easy availability, the convenient oral application route, the potent erythropoietic stimulus and the fact that validated methods for the determination of soluble cobaltous salts in human urine are not routinely included in the armoury of doping control analysis, might accentuate the doping



Scheme 2. General CID routes of deprotonated isoquinoline-3-carboxamides in negative ESI mode.

relevance of cobalt preparations. Here, anti-doping rules should also fulfil the task of protecting the athlete from exposing his/her body to hazardous risks that might not be fully evaluated by the athlete. Although the intake of cobaltous chloride is not explicitly banned by WADA, it might be classified as erythropoiesis stimulating agent (S2.1) or non-approved substance (S0) according to the 2011 prohibited list.[2] Methods for the determination of cobalt in human urine that are compatible with routine doping control procedures include a GC-MS technique[114] and a validated, quantitative flow-MS method/MS method with an LOD of 50 μg/ml.[115] Other widely used and sensitive quantitation methods are based on inductively coupled plasma mass spectrometry (ICP-MS), ICP atomic emission spectrometry (ICP-AES) and electrothermal atomic absorption spectrometry (ETAAS),[116] amongst others. Since cobalt is a naturally occurring and essential trace element[117] and can be present in the urine of healthy humans at concentrations ranging from 40 to 810 pg/ml,[118] reference and threshold values that corroborate the report of an adverse analytical finding would have to be determined. The highly sensitive LC-ICP-MS technique with detection limits down to 0.8 pg/ml[118] would be the best analytical approach for this task.

Iron chelating HIF-PHI like DFO (compound **1**) or FG-0041 (compound **2**) are not clinically relevant for the induction of erythropoiesis due to a lack of specificity for the HIF hydroxylases. Moreover, it can be assumed that the HIF-stabilising effect of the compounds and the resultant up-regulation of hypoxia-compensating target genes are compromised by the overall decrease of iron availability, leading to the impairment of other essential biological processes, like the haem biosynthesis. Additionally, the doping relevance of DFO is decreased by the low oral bioavailability of the agent and the consequent need for inconvenient injections.

Although the erythropoiesis-stimulating effect of the prototypical HIF-PHI 3,4-DHB (also known as protocatechuic acid) has not been tested in humans, it possesses the potential to be misused for ESA doping for two reasons: first, its cell permeable ethyl ester EDHB was shown to increase the exercise performance of mice,[39] and second, it is naturally occurring in the diet, for example, in fruit, nuts, vegetables, and plant-derived beverages.[119] Athletes might take this as a hint for a good tolerability of the substance. 3,4-DHB has been attributed anti-oxidant and anti-carcinogenic properties but, noteworthy, the data concerning this biological activity are contradictory[120] and opposing results even indicate promotion of tumour growth under certain conditions.[121]

Numerous analytical methods for detection of 3,4-DHB from plasma, serum, faeces, or urine samples have been described. LC-MS/MS analysis identified it as the major metabolite of cyanidin-glucosides (e.g. present in blood orange juice) in serum but was found not to be directly excreted into the urine.[122] A GC-MS study with the antispasmodic drug mebeverine (MB), however, identified 3,4-DHB as urinary MB-metabolite after enzymatic cleavage of conjugates with glucuronidase or arylsulfatase,[123] indicating that it is renally excreted as conjugated Phase II metabolite. However, the natural occurrence of protocatechuic acid complicates the differentiation between nutritional and supplemental intake of this benzoic acid derivative, potentially necessitating the introduction of threshold values and/or a discussion about the classification of naturally occurring HIF stabilizers as prohibited ESAs. The same fact applies for the rare amino acid L-mimosine, naturally occurring in the legumes of tropical plants, although several reports of toxic side effects[124] decrease the possibility of an intentional misuse for performance manipulation.

To the best of our knowledge, no analytical assays for the determination of the remaining prototypical HIF stabilizers from urine or other biological matrixes have been described in the literature. However, reference material for the experimental HIF-PHI NOG (Figure 1, compound **3**, CAS registry number: 5262-39-5), DMOG (**4**, 89464-63-1), 3,4-DHB (**5**, 99-50-3), EDHB (**6**, 3943-89-3) and L-mimosine (**7**, 500-44-7) is readily available, enabling the implementation of these compounds into routine doping control procedures. As for all of the HIF-PHI discussed here, LC-ESI-MS/MS is supposed to be the most suitable instrumental setup, since it allows chromatographic separation and ionisation of target analytes without the need for prior derivatisation. However, GC-MS(/MS) might potentially be equally suited in many cases. While the analysis of plasma or serum samples could target the intact drug molecules, more information about the metabolic fate and excretion pathway is needed for the setup of reliable detection assays from urine matrix.

GATA inhibitors

EPO gene expression is not only positively controlled through oxygen dependent binding of HIF to the enhancer region of the EPO gene, but also negatively regulated by a series of transcription factors called GATA-binding proteins (GATA). Their specific binding to the GATA gene sequence of the EPO promoter inhibit its transcriptional activity.[125] GATA inhibitors are small molecules that suppress the DNA-binding of GATA and restore the activity of the EPO gene promoter, presumably by protein-acetylation of the transcription factors.[126]

The diazepane-based GATA inhibitor K-7174 (Figure 4, compound **26**), originally designed to inhibit cell adhesion to endothelial cells,[127] was shown to reverse cytokine-induced inhibition of EPO production *in vitro* and to restore blood Hb levels in mice.[128] In a subsequent paper from the same group in co-operation with Kowa Co., Ltd, the second-generation GATA inhibitor K-11706 (structure undisclosed), was reported to be orally bioactive via the same pathway but with a 1000-fold higher potency than K-7174.[129] Strikingly, in a successive article from 2007 entitled *Does K-11706 enhance performance and why*?, Imagawa *et al*. themselves discussed the relevance of this new class of compounds as doping agents, by showing that oral administration of 3 mg/kg of K-11706 for five or eight days increased EPO, Hb and Ht values and enhanced endurance performance of mice.[130] In a recent publication from 2011, the same group studied the response of K-11706 administration on the proteomic plasma profile in comparison to rhEPO treatment or hypoxia and identified a selectively up-regulated protein (fetuin-B). It is proposed to have the potential to serve as a sensitive biomarker for the abuse of GATA inhibitors in sports. A targeted screening for GATA inhibitors like K-11706 or K-7174 by means of LC-MS/MS, the most common and presumably most sensitive and efficient approach for the detection of such compounds in modern doping analysis, was not discussed in the work by Horie *et al*. As to the best of our knowledge, no data concerning the toxicity profile of K-7174 and K-11706 were reported and neither effectiveness nor tolerability of these investigational substances was studied in human subjects.

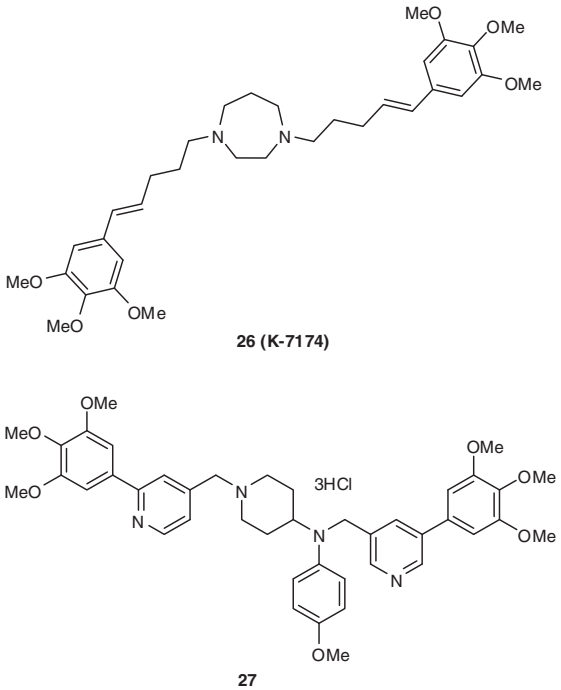


Figure 4. Molecular structures of the first-generation GATA inhibitor K7174 (compound **26**) and an exemplary follow-up product (**27**) registered for the use as erythropoiesis stimulating agent.

In a Japanese patent from 2004, published in English language as a European Patent Application in 2005, Imagawa *et al*. claimed the use of a series of polycyclic amine compounds containing either one or two 3,4,5-trimethoxyphenyl moieties as 'erythropoietin production accelerators'.[131] The experimental set-up that was used to investigate the erythropoiesis stimulating activity of the substances (e.g. the use of Hep3B cells and ICR strain mice and the dosing of 100 nM and 3 mg/kg/day for *in vitro* and *in vivo* experiments, respectively) strongly resembles the setup used in the 2004 and 2007 work by Nakano *et al*. and Imagawa *et al*., indicating that K-11706 was selected from the compounds described in this patent. The most potent inducer of EPO production amongst the discussed examples was compound **27** (4-[N-(4-methoxyphenyl)-N-[[5-(3,4,5-trimethoxyphenyl)pyridin-3-yl]methyl]amino]-1[[2-(3,4,5-trimethoxyphenyl)pyridin-4-yl]methyl]piperidine trihydrochlorid, example 13 according to the patent numbering), with effects on EPO production and haematological parameters very similar to but not exactly like the ones reported for K-11706 by Nakano *et al*. Other candidates that were reported to increase EPO production in a Hep3B cell based assay to a comparable extent and therefore also possess the potential to correspond to K-11706, could be example 114 (4-[N-(4-methylthiophenyl)-N-[[5-(3,4,5-trimethoxyphenyl)pyridin-3-yl]methyl]amino]-1[[2-(3,4,5-trimethoxyphenyl)pyridin-4-yl]methyl]piperidine trihydrochlorid, 82% of activity of **27**) or example 23 (4-[N-(3,5-dimethoxyphenyl)-N-[[2-(3,4,5-trimethoxyphenyl)pyridin-4-yl]methyl]amino]-1[[2-(3,4,5-trimethoxyphenyl)pyridin-4-yl]methyl]piperidine trihydrochlorid, 76% of activity of **27**).

As a consequence of the above mentioned physiological effects of GATA inhibitors, they can be classified as erythropoiesis-stimulating agents and are therefore prohibited according to the 2011 Prohibited List.[2] Aiming at LC-MS/MS-based analytical assays for the determination of such GATA inhibitors in sports drug testing, the piperidine core as well as the two trimethoxyphenyl moieties, each linked to a pyridine heterocycle, are conserved structural motifs, potentially providing characteristic product ions upon collision-induced dissociation of the protonated molecules. This might prove useful for the development of comprehensive LC-MS/MS screening methods for this novel class of performance-enhancing drugs in the future.

Other small-molecule ESA

PBI-1402

PBI-1402 from ProMetic Biosciences, Inc. (Laval, Canada) is an orally bioavailable, low molecular weight lipid that has been shown to stimulate erythropoiesis in humans. Although also referred to as EPO analogue, it increases red blood cell formation via an EPO-independent mechanism of action. PBI-1402 appears to promote the production and maturation of burst-forming unit-erythroids (BFU-Es), red blood cell progenitors that are not physiologically regulated by EPO. Further EPO-induced differentiation of BFU-Es into colony-forming unit-erythroids (CFU-Es) eventually leads to increased reticulocyte and erythrocyte formation. Interestingly, an additive effect of simultaneous treatment with PBI-1402 and EPO was observed in human cells *in vitro*. To date, no fully peer-reviewed work has been published on the biological effect of PBI-1402 and first-hand information is only available from posters presented by ProMetic on various haematological conferences and available for download from the company's website.[132] Moreover, a thesis dealing with the mechanism of action of PBI-1402 is obtainable from the University of Montréal[133] but the included article entitled *PBI-1402 enhances expansion of human erythroids progenitors via the Erk1/2 pathway and reduces erythropenia in myeloablated mice post transplantation* has not been published in a peer-reviewed journal.

In Phase I clinical trials with 36 healthy volunteers PBI-1402 has demonstrated a good tolerability without side-effects and significantly increased the numbers of reticulocytes after oral administration. In Ib/II trials conducted in Eastern Europe with 18 patients suffering from chemotherapy-induced anaemia, daily oral doses of PBI-1402 (44, 66 or 88 mg/kg) for 8 weeks resulted in a significant rise in Hb levels and RBC counts.[134]

In a 2004 patent application, ProMetic Biosciences published surprising results, showing that a series of medium-chain length fatty acids (MCFA) and triglycerides, well known and widely used in the pharmaceutical, cosmetic and food industry, are stimulators of erythropoiesis and claimed their therapeutic use in humans.[135] Among the presented lipid molecules, sodium caprate (Figure 5, compound **28**) and the triacylglycerides tricaprin and tricaprylin (compounds **29** and **30**, respectively) were the most prominently discussed examples. Oral administration of sodium caprate for four days significantly increased the total number of peripheral blood cells in mice. In another example, primary human bone marrow cells treated with tricaprin for five days, showed a 13-fold rise in the number of erythrocyte progenitor cells BFU-E. Moreover, ProMetic reported an anticancer activity of PBI-1402 in several pre-clinical *in vitro* and *in vivo* models and the company has filed a patent for the treatment of pancreatic cancer by the therapeutic combination of the anti-cancer drug gemcitabine with a series of MCFA and their glycerol esters.[136]

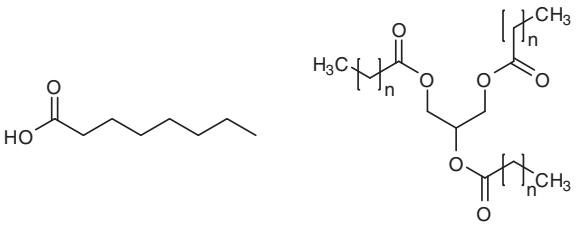


Figure 5. Medium-chain length fatty acid salts like sodium caprate (**28**) and trigylcerides thereof (**29** and **30**) were claimed to stimulate erythropoiesis.

Capric acid (C8) and caprylic acid (C10) are saturated MCFA naturally occurring in palm kernel, coconut, and milk fat.[137] They are recognized as safe additives for use in food by the FDA[138] and are readily available as nutritional supplements. Moreover, in Japan sodium caprate is used as absorption enhancer for drugs in humans.[139] The triglycerides of MCFA (MCT) are equally well absorbed by the intestine and known to be more completely hydrolysed after uptake than their long-chain analogues. Since they are hardly stored in body tissue but quickly metabolized and converted into energy, MCT are being used in sports beverages and power bars as high-density energy source and are also included in weight-loss diets.[140–142]

The dicarboxlyic acids suberic (C8) and sebacic (C10) acid are well established urinary metabolites of tricaprylin and tricaprin ingestion, respectively.[143] Diets containing high proportions of MCT oil (mainly consisting of caprylic and capric acid triglycerides) resulted in an 65- and 284-fold increased excretion of suberic acid and sebacic acid, respectively.[144] Quantification of dicarboxylic acids from urine has predominantly been achieved by GC-MS detection of derivatized target analytes, which were obtained from urine by liquid-liquid extraction. Quantitative HPLC-UV methods have also been described in the literature.[145,146]

The presence of MCFA and related MCT in various non-prohibited cosmetic, pharmaceutical and nutrition products imposes a major hurdle to the detection of an abuse of such compounds as erythropoiesis stimulating agent in sports. If PBI-1402 should in fact turn out to consist of one of the presented naturally occurring lipids, it will be indispensable to discuss, whether and how this erythropoiesis-stimulating substance is to be classified as doping agent and a potential misuse might be detected in doping control analysis.

Conclusion

The scientific progress in the field of hypoxia-regulated erythropoiesis and the report of numerous ESAs that have recently entered or advanced in clinical trials, underscore the importance of monitoring these novel emerging substances in routine doping control analysis. Stabilisation of the hypoxia-inducible transcription factor HIF is the most widespread and advanced approach of small-molecule based ESA therapies. Due to the continuously increasing number of emerging drug candidates, a targeted screening for prohibited substances becomes ever more challenging. To date, only one LC-MS/MS based analytical assay for the detection of HIF stabilizers in doping control has been reported in the literature. A profound challenge for doping analysts is the fact that most molecular structures of clinically non-approved small-molecule ESAs are not disclosed. It cannot be excluded, however, that such investigational agents are already available during the time of clinical trials, as was recently reported for the doping-relevant, emerging drugs AICAR, GW1516 and the selective androgen receptor modulator MK-2866.[147] Athletes with the hope of a performance-enhancing outcome might even participate in clinical studies, as was recently discussed on a cyclists' Internet platform upon Bayer's recruitment of healthy humans for Phase I trials of one of its HIF-PHIs. Therefore, exemplary compounds for the respective drug candidates' have been proposed and alternative non-targeted approaches of determining HIF stabilizers and other non-approved substances by different mass spectrometric scanning techniques were reviewed.

Finally, it should be noted that the illicit use of clinically non-approved substances in sports not only interferes with the spirit of fair-play but also constitutes a serious hazard for the athlete's health. The transcription factor HIF induces the expression of hundreds of target genes and 2-OG and iron dependent hydroxylases not only control the fate of HIF but are involved in numerous other signalling pathways. The effects of a long-term or unspecific modulation of the PHD-HIF axis therefore remain to be investigated. In this case, the WADA rules also have to fulfil the task of protecting the athlete from exposing his/her body to hazardous risks that are difficult or even impossible to fully evaluate. Ensuring a timely detectability in doping analysis hopefully exerts an additional deterrent influence on athletes intending to gamble with such incompletely tested emerging drugs.

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