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A Fluorescent Benzo[g]isoquinoline-based HIF Prolyl Hydroxylase Inhibitor for Cellular Imaging

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Abstract: Prolyl hydroxylation domain enzymes (PHD) catalyze the hydroxylation of the transcription factor Hypoxia Inducible Factor (HIF) and serve as cellular oxygen sensors. HIF and the PHD enzymes regulate numerous potentially tissue protective target genes which can adapt cells to metabolic and ischemic stress. We describe a fluorescent PHD inhibitor (1-chloro-4-hydroxybenzo[g]isoquinoline-3-carbonyl)-glycine which is suited to fluorescent based detection assays and for monitoring PHD inhibitors in biological systems. In cell based assays, application of the fluorescent PHD inhibitor allowed co-localization with a cellular PHD enzyme and led to live cell imaging of processes involved in cellular oxygen sensing.

Hypoxia and ischemia are central pathophysiological processes which are involved in many acute ischemic diseases such as myocardial infarction, stroke or peripheral artery disease. Groundbreaking work over the last 15 years has revealed that reduced oxygen supply not only impairs cellular function, but also activates gene expression via the hypoxia inducible transcription factors (HIF). HIF is an α/β heterodimeric transcription factor that regulates multiple cellular and systemic responses to hypoxia, including erythropoiesis, angiogenesis, altered energy metabolism, immune/inflammatory signaling, and cell differentiation/survival decisions. The two best characterized human HIF- α isoforms (HIF-1 α , HIF-2 α) have distinct, though partially overlapping, roles in these processes.^[1-5] The activity of HIF is controlled by a set of 'oxygen-sensing' 2-oxoglutarate-(2OG) and iron dependent dioxygenases: in humans three HIF prolyl hydroxylases (PHDs 1-3) and a HIF asparaginyl hydroxylase (FIH). The PHDs catalyze hydroxylation of specific

prolyl residues to promote von Hippel Lindau protein (VHL)-mediated HIF- α degradation, and FIH controls p300/CBP-promoted HIF transcriptional activity.^[1,2] The obligatory and, at least for the PHDs, rate-limiting requirement for molecular oxygen as a co-substrate renders the HIF system oxygen sensitive.^[6] Inhibition of the HIF hydroxylases by active-site binding 2OG competitors mimics hypoxia, enabling pharmacological activation of the hypoxic response in the presence of oxygen.^[1,7] Lead compounds from pharmaceutical companies are in clinical phase II and III trials and regulatory filings are anticipated in the near future for the first PHI drugs (e.g. FG-4592/Roxadustat).^[8-11]

Since many HIF-regulated genes improve tissue oxygenation or confer adaptation to hypoxia, there is clear potential to mitigate or prevent acute and chronic tissue injury through therapeutic activation of the HIF pathway. To date, exploitation of HIF stabilization by PHD inhibition has largely focused on treating anemia. However, PHD inhibition has the potential to treat a broad range of ischemic/hypoxia diseases of major unmet medical need, including vascular occlusion, stroke, inflammatory disease, and wound healing.

Substantial progress has been made in discovering the underlying basic biology of the transcription factor HIF and its cellular regulation (Figure 1).^[12-17] Alongside this work, the PHDs have been shown to be tractable drug targets. In many experimental studies proof-of-principle has been provided for the beneficial effects of cellular HIF stabilization via PHD inhibition. However, activation of the HIF system may also have adverse consequences. In order to exploit the widespread potential of HIF pathway activation via PHD inhibition, there is a need to better understand its diverse biological effects in specific

disease contexts and to design strategies for selective and restricted HIF activation.

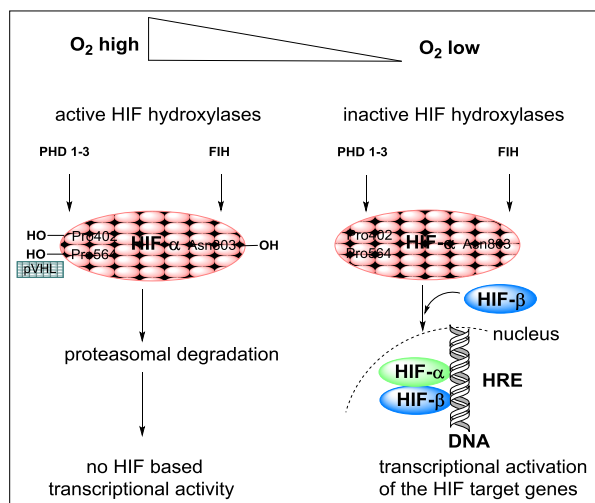


Figure 1. The role of oxygenases in O_2 sensing for the HIF system.^[1,7]

Emerging evidence points to the importance of HIF and, maybe, PHD localization, as factors in addition to their levels and post-translational modification status in regulating the hypoxic response.^[18] However, tracking the cellular processing and trafficking of PHD inhibitors, their co-localizing PHD enzymes, and of the HIF protein substrate remains difficult. Fluorescent inhibitors have potential to trace the trafficking of such inhibitors as well as of the PHD enzymes and could thus provide information regarding tissue selectivity/enzyme distribution and inhibitor selectivity. Efficient high-throughput screens and drug detection assays in sports applications could be developed using such inhibitors.

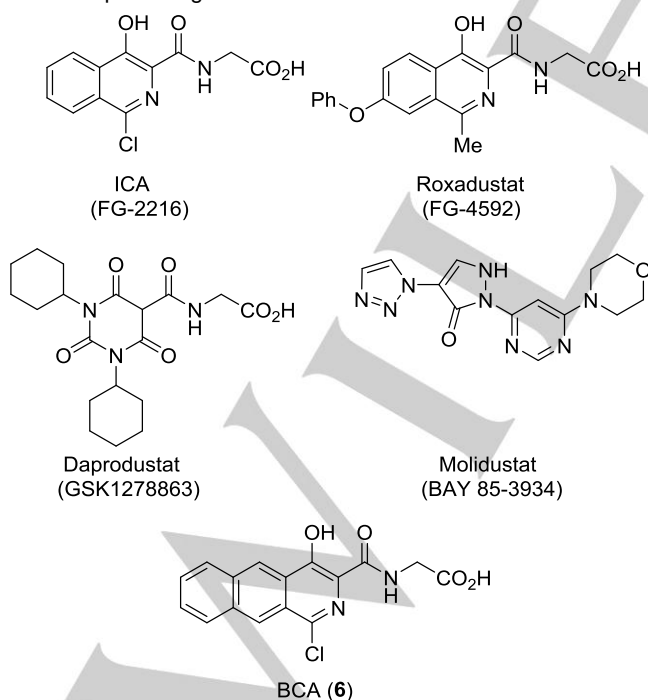


Figure 2. Examples of prominent clinically applied PHD inhibitors and the designed fluorescing PHD inhibitor BCA (**6**).

Various small-molecule PHIs, mostly analogs/competitors of 2OG, have been described (reviewed by Rabinowitz).^[19] Among these are compounds that have been, or still are, subject of clinical trials, FG-2216, FG-4592 (Roxadustat), GSK1278863 (Daprodustat), and BAY-85-3934 (Molidustat), which we considered as starting points for the development of a PHD-selective fluorescing inhibitor (Figure 2).^[20,21]

In one line of investigation, we focused on 2-(1-chloro-4-hydroxyiso-quinoline-3-carboxamido)acetic acid (ICA or FG-2216).^[13,19,20,22] Based on our experience with this isoquinoline ring system, an extended aromatic ring system, as present in (1-chloro-4-hydroxybenzo[g]isoquinoline-3-carbonyl) glycine (**6**) (BCA), was identified as a promising candidate for a PHI manifesting fluorescence properties.

In designing the new inhibitors, we focused on two considerations: Firstly, we aimed to make relatively lipophilic rather than hydrophilic PHD inhibitors for use *in vivo*. Thus, the log P_{OW} (logarithm of the octanol-water partition coefficient) of the new PHIs should be in the range of 1.5 to 3.0, as is the case for the clinically used PHIs. With a calculated log P_{OW} value of 2.94 (see ESI), compound **6** lies in this range. Secondly, the modification should still allow for tight binding to the PHD active site. Our biophysical studies on PHD2 have revealed a tight and relatively rigid binding site for 2OG but that HIF-1 α substrate binding involves both hydrophobic and polar interactions and a flexible loop ($\beta 2\beta 3$ loop) that encloses the active site in the enzyme-substrate complex.^[23,24] Hence, we postulated that the known ICA inhibitor could be extended in the direction pointing out of the active site in the region occupied by HIF- α substrate. The proposed binding mode of **6** was supported by semi-automatic docking with PHD2 (Figure 3) (details of the docking and energy-minimization procedure are given in the ESI).

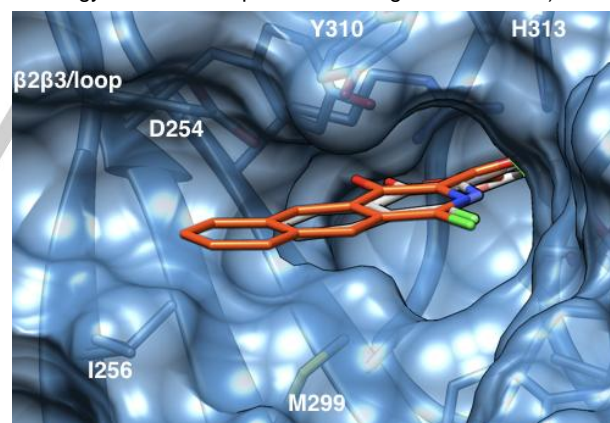
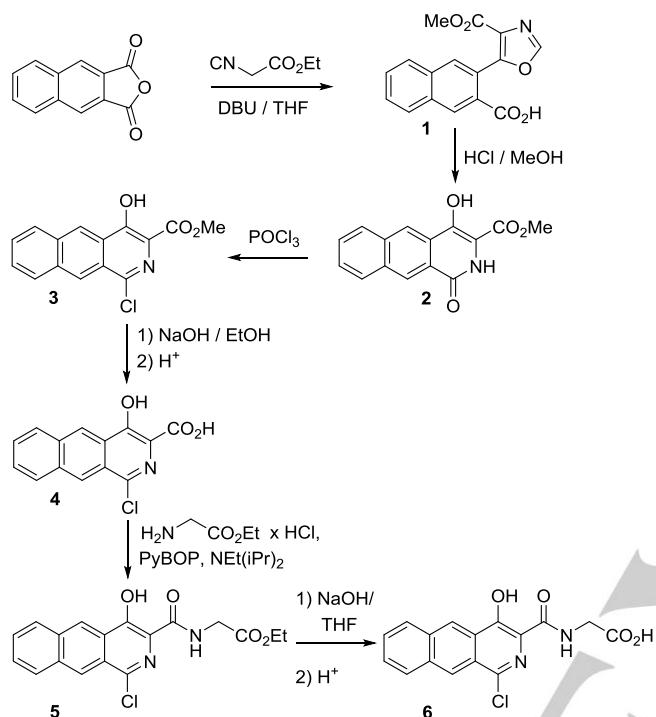


Figure 3. Putative protein-inhibitor interactions of BCA (**6**, orange sticks) compared to the crystal structure (PDB-ID: 2HBT) with FG-2216 (ICA, sticks gray). The solvent-excluded surface indicates the entrance to the active site pocket.

For the six-step synthesis of **6** from 2,3 naphthalene dicarboxylic acid anhydride, the established route for isoquinoline-based PHD inhibitors was applied (Scheme 1).^[25-27] Reaction between methyl isocynoacetate and DBU yielded 3-(4-(methoxy-carbonyl)oxazol-5-yl)-2-naphthoic acid (**1**). Acidic rearrangement and decarboxylation resulted in methyl 4-hydroxy-1-oxo-1,2-dihydrobenzoisoquinoline-3-carboxylate (**2**) and subsequent reaction with phosphoryl chloride in methyl 1-chloro-4-hydroxybenzo[g]isoquinoline-3-carboxylate (**3**). Saponification, protonation, and amide-coupling using glycine

ethyl ester and PyBOP yielded ethyl (1-chloro-4-hydroxybenzo[g]isoquinoline-3-carbonyl)glycinate (**5**), which was purified by column chromatography. In the final step, alkaline ester hydrolysis and protonation resulted in the desired inhibitor (1-chloro-4-hydroxybenzo[g]isoquinoline-3-carbonyl)-glycine (**6**), in an overall yield of 14 % from **1**. Although inhibitor **6** seems to be reported in a patent,^[28,29] to the best of our knowledge no synthetic procedure or experimental data has been available for it.



Scheme 1. Synthesis of the benzo[g]isoquinoline inhibitor BCA (**6**).

Solid BCA (**6**) can be stored for months at ambient conditions and a solution of **6** in DMSO-*d*₆ showed no degradation according to the ¹H NMR data after incubation for 24 h at 37° C. The excitation and absorption spectra of BCA (**6**) in methanol are characterized by three maxima at λ = 250, 285 and 380 nm (absorption); 250, 305, and 380 nm (excitation). When excited at λ = 380 nm, an intense fluorescence centered at λ = 504 nm was observed (Figure 4).

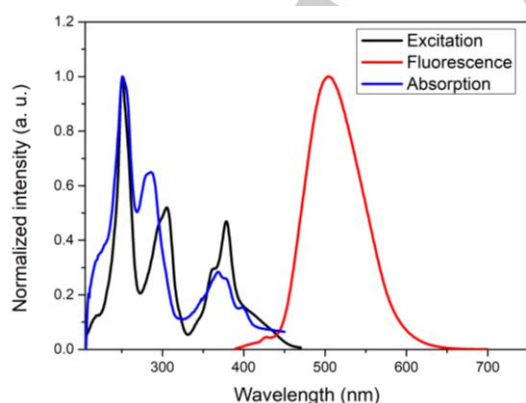


Figure 4. Absorption, excitation and emission spectra of **6** (methanol).

The half-maximal inhibitory concentration (IC₅₀) of BCA was measured using a MALDI-TOF MS inhibition assay. The PHD2 assay consisted of PHD2, Fe(II), ascorbate, 2OG and HIF-1α CDD peptide in HEPES with varying concentrations of inhibitor. The IC₅₀ value for PHD2 was determined to be 1.10 ± 0.21 μM, indicating that BCA is a reasonably potent inhibitor (Figure 5).

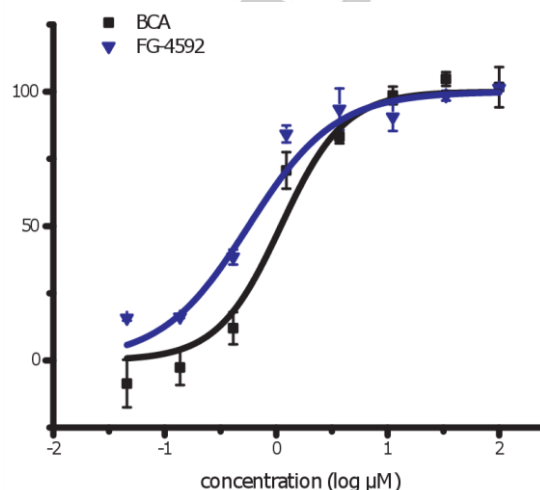


Figure 5. Determination of the IC₅₀ value of BCA (**6**); IC₅₀ (PHD2) = 1.10 ± 0.21 μM. Assay in HEPES 50 mM at pH 7.5 (PHD2 1 μM, Fe(II) 10 μM, 2OG 60 μM, ascorbate 100 μM, HIF-1α-CDD 50 μM, DMSO 1%).

The binding of BCA (**6**) to PHD2 was confirmed using nuclear magnetic resonance spectroscopy, which show that BCA is a moderate to strong binder of PHD2. The addition of BCA (**6**) to PHD2 leads to a line broadening of the BCA peaks, which is characteristic of binding (Figure 6) (see ESI).

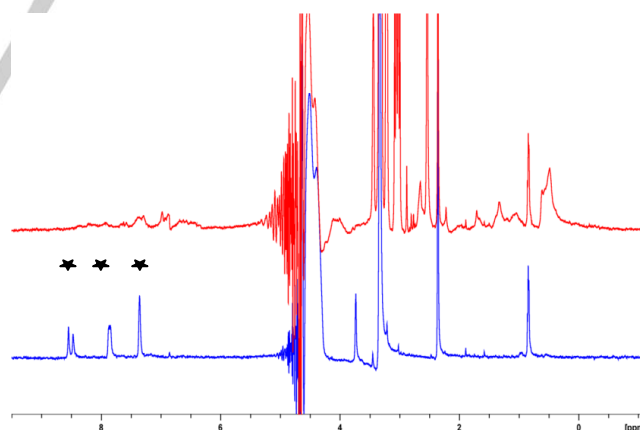


Figure 6. ¹H excitation sculpting NMR spectra of BCA (**6**) (lower trace, blue) and PHD2 with BCA (**6**) (upper trace, red). Stars (*) indicate the peaks corresponding to BCA (**6**).

To study the selectivity of BCA, the inhibitor properties were also tested with representative other human 2OG dependent dioxygenases, *i.e.* the lysine specific demethylases KDM5B (JARID1B) and KDM6B (JMJD3) as well as the asparaginyl hydroxylase FIH. AlphaScreen methodology was used for the lysine demethylases and RapidFire MS was used for FIH, as

previously reported^[7,11,30-32] The results, IC_{50} values resulting in IC_{50} (KDM5B) = 34.3 μ M, IC_{50} (KDM6B) > 100 μ M and IC_{50} (FIH) = 78.4 μ M, indicate that BCA is, at least partially selective for the PHDs over other human 2OG oxygenases.

Western blot analyses were performed to obtain information about the ability of BCA (6) to inhibit the PHDs in cells and so stabilize HIF (Figure 7). HeLa cells were incubated with BCA (6) for four hours. Accumulation of HIF-1 α was observed with a maximum at an inhibitor concentration of 500 μ M, the highest concentrations that could be applied due to the solubility of BCA (6) in the buffer/DMSO mixture.

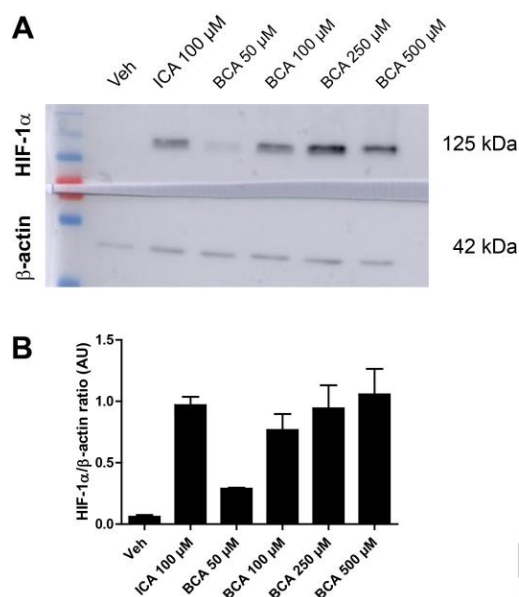


Figure 7. HeLa cells were incubated using DMSO as a vehicle (Veh) with ICA or BCA (6) at the indicated concentrations for 4 h. (A) Representative immunoblot analysis of cell lysates with antibodies against HIF-1 α and β -actin. (B) Densitometric quantification of immunoblots from 3 independent experiments. Values are expressed as ratio of HIF-1 α to β -actin signals and presented as mean \pm SEM in arbitrary units (AU).

Since our research focus partially lies on preventing acute and chronic kidney tissue injury and renal failure by PHD inhibitors, more specific studies to stabilize HIF-1 α were performed by western blotting using Immortalized Human Kidney Epithelial (IHKE) cells. IHKE cells were incubated with BCA for 5 hours resulting in a significant HIF-1 α stabilization at concentrations about 100 μ M BCA (Figure 8).

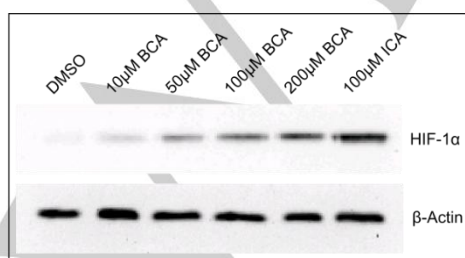


Figure 8. Immunoblot analysis of IHKE cell lysates with antibodies against HIF-1 α and β -actin. IHKE cells were incubated with BCA for 5 hours. β -actin served as loading control.

It is worthwhile mentioning that IHKE cells lack (after some generations) organic anion transporters (OATs).^[33] Thus, no such transporter for cellular uptake is needed, hence BCA uptake likely occurs via passive diffusion due to its lipophilicity. As shown in previous studies, lipophilic 2OG oxygenase inhibitors such as ICA do not rely on the uptake via a transporter.^[13,34] In contrast, hydrophilic inhibitors including 2,4-pyridinedicarboxylic acid (PDCA) are transferred via the organic anion transporter (OATs).^[13,34] BCA was injected into mice 2.5 hours before kidney removal to test for HIF-1 α stabilization *in vivo*. Strong nuclear HIF-1 α signals were detected in epithelial cells of all tubular segments, while control kidneys showed nuclear HIF-1 α positivity only in isolated tubular cells of the distal nephron (Figure 9).

These observations clearly indicate the *in vivo* cellular uptake as well as the *in vivo* stability of BCA and suggest that BCA/related compounds might be useful tools for studies on pharmacokinetics and pharmacodynamics.

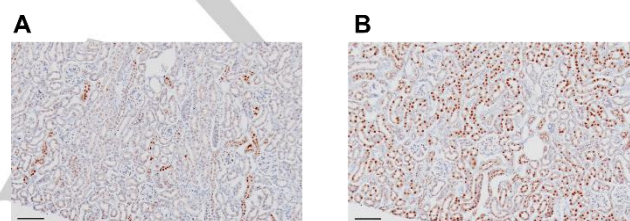


Figure 9. Immunohistochemistry for HIF-1 α in kidney sections. (A) Vehicle treated kidney section (0.5 M Tris buffer, DMSO 10%). (B) Strong nuclear HIF-1 α signals in epithelial cells of all tubular segments. BCA (40 mg/kg, i.e. 1 mg) was injected intraperitoneally 2.5 hours before kidney removal. Scale bar: 100 μ m.

Figure 9. Immunohistochemistry for HIF-1 α in kidney sections showed a strong signal in nuclei of tubules. Mice were injected once with BCA 2.5 h before kidney removal. Scale bar: 50 μ m.

To investigate whether BCA (6) is suitable for fluorescence microscopy, HeLa cells were incubated with a concentration of BCA sufficient to stabilize HIF-1 α for 4 h. BCA (6) showed readily detectable fluorescence signals when excited at appropriate wavelengths. Although the signal could be detected in the whole cell, it accumulated around the nuclei. Staining for PHD2 by immunofluorescence indicated a co-localization of BCA (6) and PHD2 close to the nuclei. This observation implies that BCA is selectively bound to PHD2, which is mainly localized around nuclei (Figure 10).^[35]

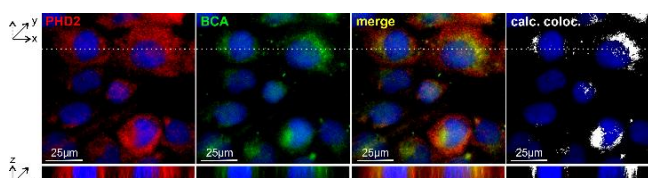


Figure 10. HeLa cells were incubated with 100 μ M BCA (6) (green) for 4 h and stained for PHD2 by immunofluorescence (red) and for nuclei using DAPI (blue). Fluorescence signals of cytoplasmic PHD2 protein and BCA (6) were merged (yellow). Co-localization of PHD2 and BCA was calculated and highlighted in white. The upper row of images represents xy-planes, the lower row the corresponding xz-planes along the white dotted line.

In summary, an efficient six-step synthesis leads to the isoquinoline-based PHD inhibitor BCA (6) which has fluorescent

properties and good PHD2 binding activity. BCA (6) is suitable for fluorescence microscopy and is useful as a tool to assay the distribution of PHD complexes in cells. Inhibitors following the concept underlying BCA (6) will help to provide improved mechanistic understanding of HIF activation and the selectivity of PHD inhibitors in order to optimize therapeutic strategies. The concept will also be useful for future studies on the pharmacodynamics and pharmacokinetics of PHIs and should be adaptable to the study of other oxygenases.

Experimental Section

Synthetic procedures, NMR data and spectra, NMR binding experiments, determination of the IC₅₀ value, spectral characterization as well as details of the docking studies, log P_{OW} calculations, immunoblotting, immunohistochemistry and immunocytochemistry are provided in the Supporting Information.

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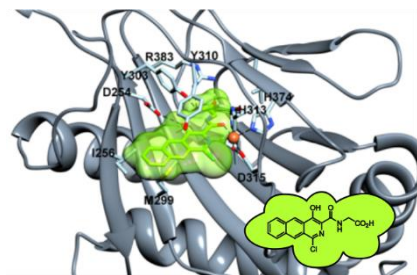
Keywords: HIF system • PHD inhibitor • hypoxia • oxygen sensing • fluorescence microscopy

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Entry for the Table of Contents



A potent benzoisoquinoline-based HIF prolyl hydroxylase inhibitor (HIF-PHI) is described. The fluorescence features of the compound permit its application to HIF prolyl hydroxylase imaging in cells.