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Hypoxia-inducible factor (HIF) pathway as target for doping purposes and mass spectrometric characterisation of orally available model HIF-stabilizers

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Introduction

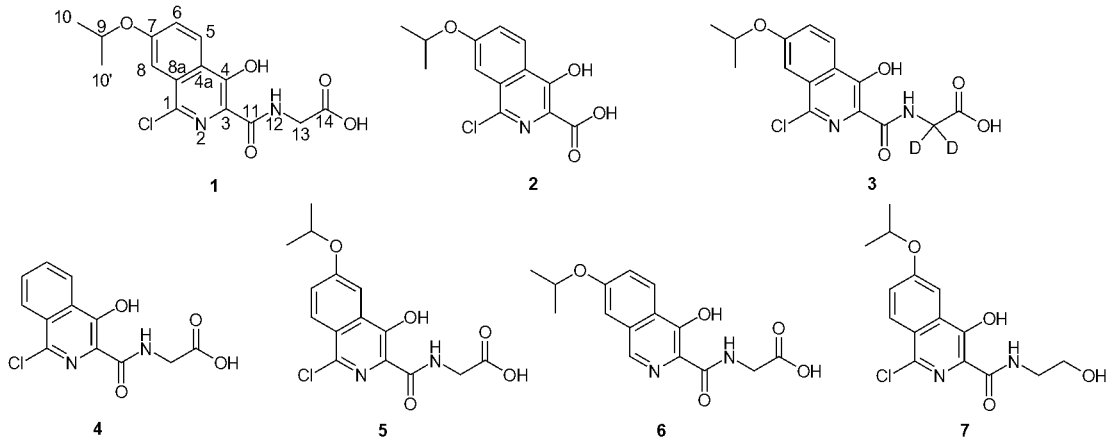
The development of new, effective and convenient therapies against anaemic disorders has brought up a number of novel drugs in the past years, some of which are already commercially available, like numerous biosimilar epoetins or the continuous erythropoiesis receptor activator CERA, while many others are still under clinical investigation, e.g erythropoietin-mimetics like Hematide [1]. Recently, after its central role in molecular oxygen sensing has been unravelled, one important pivot point for the pharmacological regulation of endogenous erythropoiesis has become the hypoxia inducible factor (HIF) pathway. HIF is a heterodimeric transcription factor composed of α- and β-subunit [2]. While the latter is constitutively expressed, the stability of HIFα is strongly dependent on oxygen tissue concentration. In normoxia, posttranslational hydroxylation of two distinct proline residues in the oxygen-dependent degradation domain of HIFα induces its rapid proteasomal degradation. Hypoxic conditions, where less oxygen substrate is available for hydroxylation, suppress the proline modification which consequently leads to a stabilisation and accumulation of HIFα. Translocated to the nucleus, where it dimerises with HIFβ and other co-factors, it stimulates the transcription of genes for hypoxia compensating processes, such as angiogenesis, erythropoiesis and glucose metabolism [3]. A family of 2-oxoglutarate and iron dependent enzymes, the prolyl hydroxylase domain proteins (PHD), are specific catalysts

for the hydroxylation of HIF-prolyl residues and, as their activity was shown to be directly oxygen controlled, can be termed the molecular oxygen sensors of the mammalian cell [4]. Various approaches for addressing and activating the HIF pathway have been developed and recently patented, such as gene transfer of constitutively active HIFα with specific oligonucleotide sequences, peptides inhibiting the ubiquitination and degradation of HIF or mutant HIFα polypeptides in which hydroxylable amino acids are modified [5]. However, the most promising approach in terms of clinical application is the xenobiotic inhibition of PHD activity by small molecule drugs (prolyl hydroxylase inhibitors, PHI) that have been designed and tested in large scale regarding their ability to induce the erythropoietic cascade [6, 7]. Fibrogen (South San Francisco, CA) has protected by patent a great number of heterocyclic carboxamides, hydroxamates and phenantrolines for use as potentially therapeutic HIF-PHI [8]. Their lead drug candidate FG-2216 is an orally available anti-anaemia agent currently in phase 2 clinical trials that was shown to raise blood haemoglobin (Hb) levels in CKD (chronic kidney disease) patients by up to 2.4 g/dL after 15 weeks of oral administration [9]. Remarkably, low dosage of the drug led to significant Hb-rise despite only modest elevation of plasma erythropoietin (Epo) levels, indicating the stimulation of the complete erythropoietic machinery, including increased expression of genes for red blood cell maturation and enhanced iron metabolism (e.g. Epo receptor, transferrin and its receptor, ferroportin, and the divalent metal transporter 1) [10]. After one case of death by fulminant hepatitis caused a clinical hold of the trials of FG-2216 and FG-4592, another anti-anaemia drug candidate, the FDA permitted to resume clinical testing in April 2008 [11, 12].
Since this novel class of compounds is capable of enhancing the organism's capacity for molecular oxygen transport, it also possesses great potential for abuse as performance enhancing agent in sports. HIF stabilisers might be prohibited according to the WADA prohibited list 2010 (M3.2) as gene doping agents that alter gene expression. Preventive doping research requires the timely development of analytical assays for substances emerging from current clinical trials, if possible even before their entering the market as approved drug. Aiming at analytical method design, we studied the mass spectrometric dissociation behaviour of 5 isoquinoline-3-carboxamides [13, 14] that might potentially be clinically relevant HIF stabilisers according to literature data and patent specifications.

Experimental

Synthesis and characterisation of model compounds

The lead model PHI and related compounds (Figure 1, compounds **1**-**3**) as well as four structural analogues (compounds **4**-**7**) were synthesized in accordance to methods described elsewhere [15]. All structures were confirmed by high field nuclear magnetic resonance spectroscopy as well as by determination of the elemental compositions using high resolution/high accuracy mass spectrometry utilizing a LTQ Orbitrap (Thermo, Bremen, Germany) at a resolving power of 30,000 (FWHM) [13, 14].



Electrospray ionization-tandem mass spectrometry

ESI-MS(/MS) experiments were conducted on a LTQ Orbitrap mass spectrometer. Spectra were recorded in positive ionization mode after calibration was achieved with the manufacturer's calibration mixture. Mass accuracies <5ppm were monitored and accomplished for the period of analysis. Analytes were dissolved in acetonitrile/water (1:1, v/v) containing 0.5% of formic acid at concentrations of 10 µg/mL and introduced into the ion source of the mass spectrometer with a syringe pump at a flow rate of 5 µL/min. The ionization voltage was set to 4200 V, the capillary temperature was 275 °C and for MS^n experiments the precursor ions were isolated with a width of 1.5 Da. The protonated species were dissociated at normalized collision energies between 12 and 30 arbitrary units (Xcalibur software version 2.0, Thermo, Bremen, Germany). Damping and collision gas in the linear ion trap was helium (purity grade 5.0), while nitrogen, obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany), was used for the curved linear ion trap (CLT).

Additional ESI-MS(/MS) studies were performed using Applied Biosystems API 2000 and and API 4000 QTrap mass spectrometers (Darmstadt, Germany) with electrospray ionization and direct infusion of the analyte working solutions. Nitrogen was employed as curtain and collision gas.

Density functional theory calculations

For additional support of the proposed dissociation schemes, density functional theory calculations were conducted at the PBE /def2-TZVP level of theory using TURBOMOLE 5.9 [16] to yield molecular geometries as well as molecular and reaction energies. Proton affinities are computed as the differences between the total energies of the neutral reactant and the cation without vibrational or thermal contributions.

In-vitro metabolism studies

Metabolites were identified and synthesised in an *in-vitro* assay using a substrate concentration of 100 µM, 0.2 mg/mL pooled human liver enzymes (S9 and microsomal fraction from BD Biosystems, Woburn, MA) and 5mM NADPH in phosphate buffer, pH 7.4 with 5 mM MgCl2. The samples were incubated in a total volume of 100 µL for 2 h at 37 °C before the reaction was stopped by addition of 100 µL of cold acetonitrile. The LC-MS/MS analysis of liquid-liquid extracts (ethyl acetate) was carried out using an Agilent 1100 Series liquid chromatograph equipped with a Xterra C18 column (particle size 3.5 µm, 2.1 x 150 mm; Waters, Milford, MA) and interfaced to an Applied Biosystems API 4000 QTrap mass spectrometer. A standard gradient using 5 mM ammonium acetate containing 0.1% of acetic acid (mobile phase A) and methanol (mobile phase B) was applied at a flow rate of 200 µL/min and an analysis time of 27 min.

Results and Discussion

Mass spectrometric information required for the development of analytical assays for HIF stabilisers with isoquinoline scaffold was gained by studying the collision induced dissociation behaviour of the five protonated model HIF-PHI **1** and **4-7** (Figure 1). Herein, we exemplary discuss the CID of compound **1** in detail. For further information see [13, 14]. Density functional theory calculations of proton affinities at isoquinoline nitrogen (Figure 1, N-2) and amide oxygen (CO-11) were conducted. Assuming a conformationally flexible glycine amide sidechain in an excited state of the molecule, protonation at N-2 was found to

be energetically favoured by 3.8 kcal/mol. However, several stabilising hydrogen bonds can theoretically be formed, resulting in almost isoenergetical protonation affinities at N-2 and amide oxygen CO-11. The further discussion is based on N-2 protonated isoquinoline.

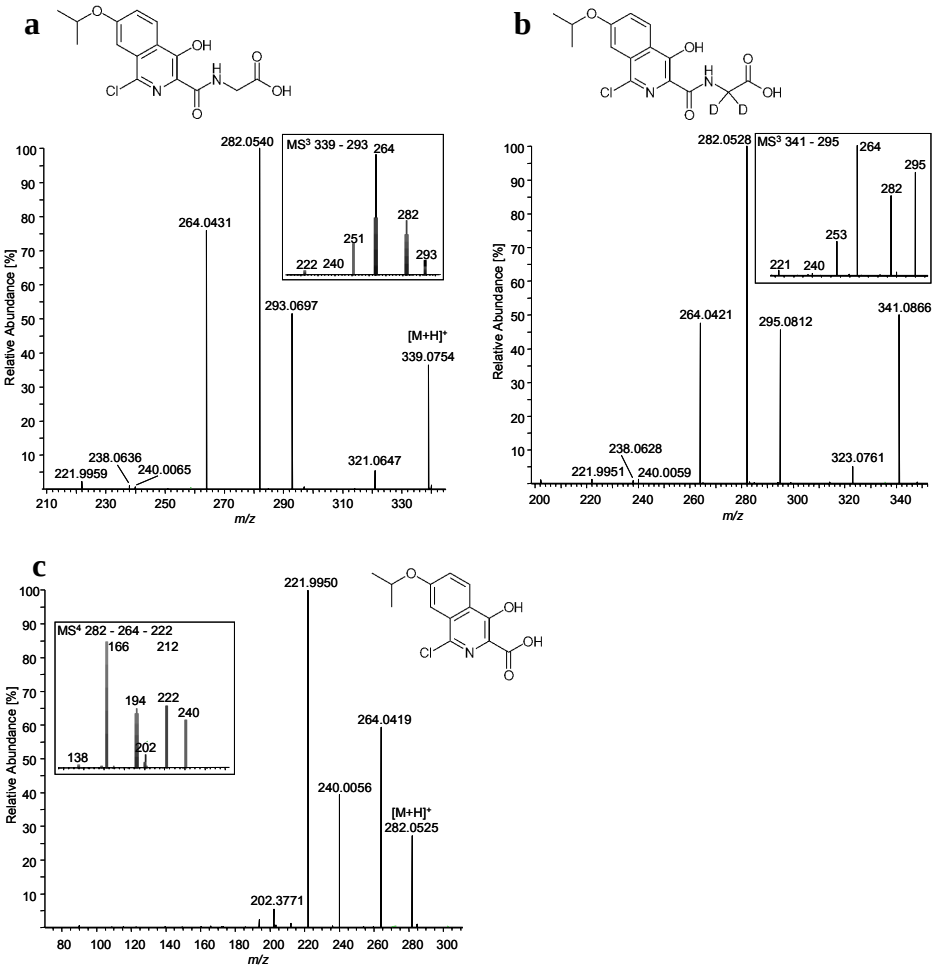


Figure 2. ESI product ion spectra (LTQ-Orbitrap) of protonated (**a**) Compound **1**, collision energy (CE) = 25 arbitrary units (au), MS^3 experiment CE = 19 au; (**b**) Compound **3**, collision energy = 25 au, MS^3 experiment CE = 18 au; (**c**) Compound **2**, collision energy = 25 au, MS^4 experiment CE = 15 au.

Low energy collisional activation of protonated N-[(1-chloro-4-hydroxy-7-isopropoxy-isoquinolin-3-yl)carbonyl]glycine results in consecutive losses of water (–18 u) and carbon monoxide (–28 u) giving rise to the anhydride at *m/z* 321 and the isoquinoline-3-carboxylic acid methyleneamide cation at *m/z* 293. This was substantiated by determination of elemental compositions of precursor, product ions and cleaved neutral species with accurate mass measurements as well as by H/D-exchange experiments. The resulting precursor at *m/z* 343, in which three mobile protons were exchanged by deuterium and one deuteron was added during the ionisation from D_2O/MeOD solution, showed a nominal loss of 20 u due to D_2O

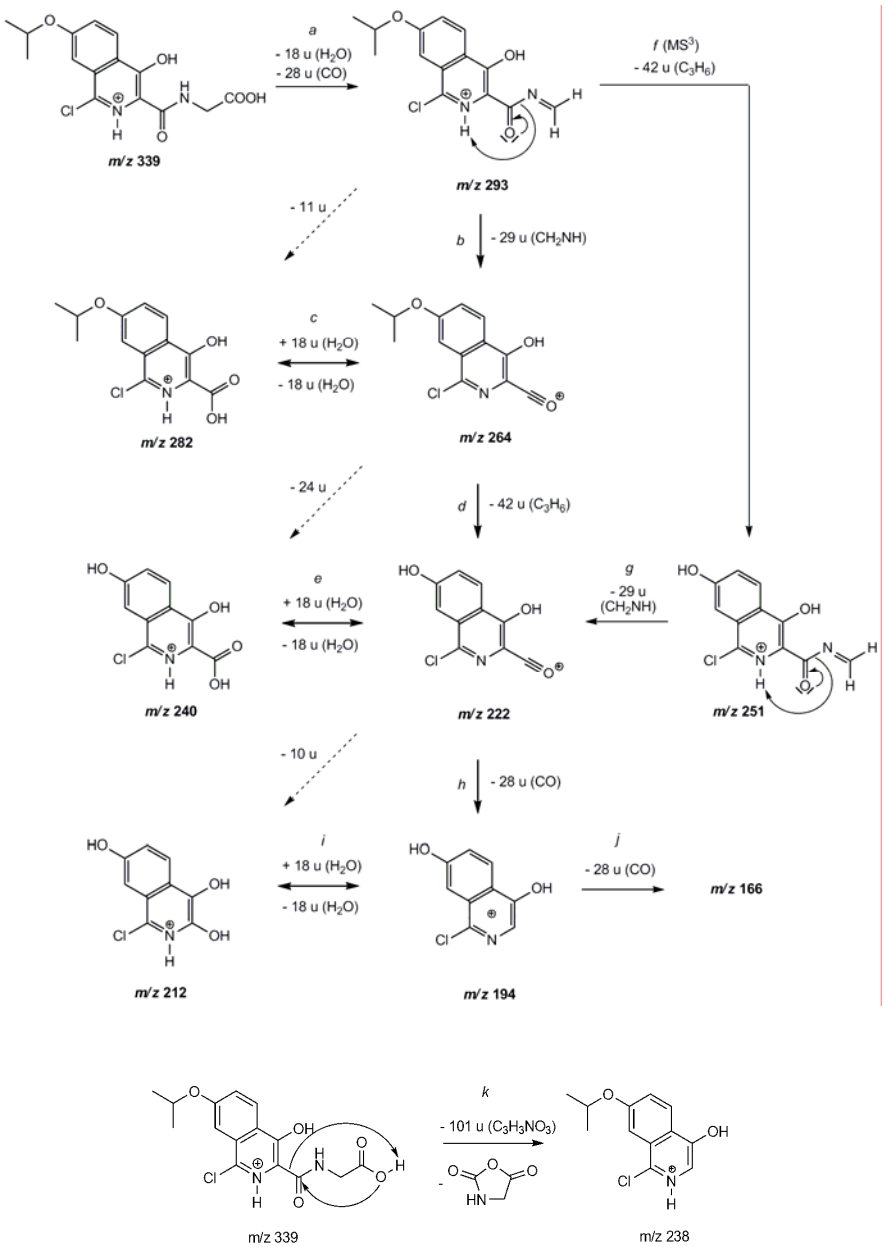
elimination (data not shown). Besides the losses of water and carbon monoxide, the protonated precursor ion $[M+H]^+$ generates a fragment ion at *m/z* 238 by the elimination of $C_3H_3NO_3$ (–101 u). As explanation for this we propose a mechanism by nucleophilic attack of the carboxylic oxygen at the amide carbon, concomitant hydrogen transfer, and final elimination of 2,5-oxazolidinedione to yield the protonated 1-chloro-4-hydroxy-7-isopropoxy-isoquinoline cation (Scheme 1, pathway *k*).

As verified by means of MS^3 and MS^4 experiments, the abundant ion at *m/z* 282 was shown to originate from the precursor at *m/z* 293, corresponding to an unusual mass shift of –11 u, and, according to the elemental composition of the product ions, to a nominal loss of HCN, concerted with the addition of an oxygen atom. Simultaneously, the fragment ion at *m/z* 293 eliminates methyleneamine (–29 u) to yield the product ion at *m/z* 264, which spontaneously adds a water molecule (+18 u) to create the same product ion at *m/z* 282. HCN elimination and instantaneous oxygen addition had earlier been proposed to occur via a putative reactive carbene intermediate.

In order to unequivocally identify the definite dissociation pathway, an isotope labelled analogue of **1** bearing two deuterium atoms at C-13 was synthesized and analyzed (compound **3**, Figure 1 and 2b). The doubly deuterated and protonated precursor at *m/z* 341 consecutively eliminates water (–18 u) and carbon monoxide (–28 u) to yield the product ion at *m/z* 295 still including both deuterium atoms and further corroborating the methylene amide structure postulated above for unlabeled compound **1** (Scheme 1, *m/z* 293). MS^3 experiments of the labelled ion at *m/z* 295 reveal that both deuterium labels are eliminated from the methylene amide precursor and the same product ions as in the spectrum of the unlabelled compound **1** are observed (*m/z* 282, 264, 240 and 222, Figure 2b). Isolation of the ion at *m/z* 264, generated by MS^2 or MS^3 fragmentation of the precursors at *m/z* 341 or 295, respectively, also yields the same water adduct at *m/z* 282 as compound **1**. Together, these findings clearly exclude the dissociation mechanism via the reactive carbene intermediate in favour of the methyleneamine elimination pathway. As proposed in Scheme 1, hydrogen transfer from the protonation site to the nitrogen atom of the leaving group and subsequent elimination of methyleneamine generates the isoquinoline-3-acylium ion at *m/z* 264, which reversibly (*vide infra*) associates a water molecule to produce the isoquinoline-3-carboxylic acid cation (*m/z* 282). Hence, the nominal loss of 11 u is suggested to be actually composed of a methyleneamine elimination (–29 u) and an instantaneous water addition (+ 18 u) via an acylium ion intermediate. Further MS^3 experiments with protonated compound **1** taking the ion at *m/z* 293 as second precursor generate the 1-chloro-4,7-dihydroxy-isoquinoline product

ion at *m/z* 251 (*m/z* 253 for d_2-labelled **3**) by elimination of propene (–42 u, Scheme 1). This ion, only observed under MS^n (n>2) conditions, shows a similar dissociation pattern as its precursor at *m/z* 293 with nominal losses of 11 u and 29 u yielding the product ions at *m/z* 240 and 222, respectively (MS^4, dealkylated analogues to *m/z* 282 and 264, Scheme 2).

As reported earlier, the water association in the gas phase of the mass spectrometer appeared to be highly reversible, allowing for MS^n "ping-pong" experiments to be conducted in the linear ion trap, where storage of the isoquinoline-3-acylium ion spontaneously produces the carboxylic acid as water adduct (Scheme 1, *c*, also *e, i*)



Scheme 1. Proposed dissociation pathways of protonated drug candidate **1**.

Upon excitation, this ion re-eliminates the water molecule to restore the initial anhydride ion structure (data not shown). The inability to fully decompose the ion at *m/z* 282 even by application of high excitation energies (100 eV) further substantiates the high spontaneousness of the acylium ion-water association and explaines the favoured gas phase ion structure of the isoquinoline-3-carboxylic acid.

MS^4 experiments proved that the isoquinoline-3-acylium cation at *m/z* 222 gives rise to the fragment ions at *m/z* 194 and 166 by consecutive losses of two molecules of carbon monoxide (– 28 u, – 28 u), as well as to the ion at *m/z* 212, corresponding to another unusual nominal loss of 10 u (–"C"/+"2H"). Similar to the behaviour of the carboxylic acid cations at *m/z* 264 and 222, the product ion at *m/z* 194, upon storage in the ion trap, adds a water molecule (+18 u) to yield the ion at *m/z* 212 with the same elemental composition as the one mentioned above. As proposed in Scheme 1, the water adduct species at *m/z* 212 probably consists of protonated 1-chloro-3,4,7-trihydroxy-isoquinoline, which might be formed by water association to the 1-chloro-4,7-dihydroxy-isoquinoline-3-carbenium ion at *m/z* 194.

The proposed gas phase structures shown in Scheme 1 could additionally be substantiated by the analysis of chemically synthesised 1-chloro-4-hydroxy-7-isopropoxy-isoquinoline-3-carboxylic acid (compound **2**, Figure 1) constituting the proposed gas phase structure of the water adduct ion at *m/z* 282 which generates the same product ions in offline ESI-MS^n experiments as the protonated compound **1** (Figure 2c).

The observed phenomena proved to be independent from ion trapping conditions and the instrumental design of the mass spectrometer. Analysis of the compounds on Applied Biosystems API 2000 and API 4000 QTrap mass spectrometers (Darmstadt, Germany) with positive electrospray ionization and direct infusion of the analyte working solutions did not yield any remarkable differences in the dissociation behaviour.

However, despite laborious efforts, the source of the gas phase water responsible for the unusual observations remained unclear. Neither the initially cleaved water molecule nor the ionization process can be the origin of the water addition, as could be substantiated by H/D-exchange experiments and the use of deuterium oxide as infusion solvent. Attempts to exchange potential residual water in the Orbitrap as well as the QTrap mass spectrometer by direct infusion of D_2O/MeOD at an average flow rate of 5 µL/min for 72 hours did not affect the masses of the relevant fragment ions.

The detailed information about the dissociation behaviour of isoquinoline-3-carboxamides not only enables the establishment of comprehensive screening procedures but also allows the

structural elucidation of *in-vitro* metabolites. Figure 3 shows the enhanced product ion spectrum of a protonated metabolite of compound **1** with *m/z* 355, corresponding to a mass shift of +16 u with respect to the parent compound. Accurate mass measurements proved the metabolic addition of an oxygen atom. This could theoretically be due to either aliphatic or aromatic hydroxylation or N-oxidation. Taking into account its dissociation pattern, the monooxygenation of **M1** unequivocally corresponds to hydroxylation of the aliphatic isopropoxy residue, because the formerly observed elimination of 42 u (corresponding to propene) is replaced by a neutral loss of 58 u from the product ion at *m/z* 280. The characteristic ion transitions of –11 and –10 u can be observed as well as the diagnostic product ions in the lower mass region of the spectrum (*m/z* 240, 222, 212, 194).

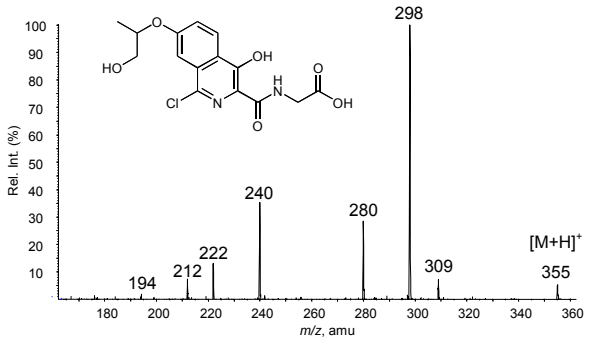


Figure 3: Mass spectrum and molecular structure of hydroxylated *in-vitro* phase 1 metabolite **M1**of HIF stabiliser **1**.

The mass spectrometric investigation of compounds **4-7** further corroborated the dissociation pathway suggested for substance **1**, as the accordant product ions and ion transitions were also observed for the structurally related isoquinoline-3-carboxamides shown in Figure **1**. Hence, the phenomena of spontaneous water addition and unusual nominal losses like 11 u and 10 u can be utilized for the screening for HIF stabilisers with isoquinoline-3-carboxamide core structure with variable substitution pattern in clinical, forensic and doping control analysis. This enables the detection of unknown potential metabolites and a wide variety of structural analogues that might emerge from the ongoing clinical development of prolylhydroxylase inhibitors as novel next generation drug candidates.

Acknowledgements

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References

1. Bunn, H. F. (2007) New agents that stimulate erythropoiesis. *Blood* 109, 868-873.
2. Wang, G. L.; Semenza, G. L. (1995) Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* 270, 1230-1237.
3. Bruick, R. K.; McKnight, S. L. (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294, 1337-1340.
4. Kaelin, W. G., Jr.; Ratcliffe, P. J. (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell* 30, 393-402.
5. Nangaku, M.; Kojima, I.; Tanaka, T.; Ohse, T.; Kato, H.; Fujita, T. (2006) Novel drugs and the response to hypoxia: HIF stabilizers and prolyl hydroxylase. *Recent Pat Cardiovasc Drug Discov* 1, 129-139.
6. Bruegge, K.; Jelkmann, W.; Metzen, E. (2007) Hydroxylation of hypoxia-inducible transcription factors and chemical compounds targeting the HIF-alpha hydroxylases. *Curr Med Chem* 14, 1853-1862.
7. Tanaka, T.; Nangaku, M. (2009) Drug discovery for overcoming chronic kidney disease (CKD): prolyl-hydroxylase inhibitors to activate hypoxia-inducible factor (HIF) as a novel therapeutic approach in CKD. *J Pharmacol Sci* 109, 24-31.
8. Arend, L.; Flippin, L.; Guenzler-Pukall, V.; Ho, W.; Turtle, E.; Du, X., *US2004025421 5A1*. 2004.
9. Provenzano, R.; Fadda, G.; Bernardo, M.; James, C.; Kochendoerfer, G.; Lee, T.; Neff, T.; Ellison, R. H.; Piper, B. A. (2008) FG-2216, A Novel Oral HIF-PHI, Stimulates Erythropoiesis and Increases Hemoglobin Concentration in Patients with Non-Dialysis CKD. *American Journal of Kidney Diseases* 51, B80.
10. Klaus, S. J.; Langsetmo, I.; Neff, T.; Lin, A.; Liu, D. (2008) Beneficial Pharmacodynamic Effects Resulting from 'Complete Erythropoiesis' Induced by Novel HIF Prolyl Hydroxylase Inhibitors FG-2216 and FG-4592. *J Am Soc Nephrol* 19, 524A.
11. Inc., A. P. (2007) Adverse event of FG-2216 for the treatment of anemia. *www.astellas.com/en/corporate/news/pdf/080402_eg.pdf* accessed 05-27-2009.
12. Inc., A. P. (2008) The FDA Accepts the Complete Response for Clinical Holds of FGH-2216/FG-4592 for the Treatment of Anemia. *www.astellas.com/en/corporate/news/pdf/080402_eg.pdf* accessed 05-27-2009.
13. Beuck, S.; Schwabe, T.; Grimme, S.; Schlorer, N.; Kamber, M.; Schanzer, W.; Thevis, M. (2009) Unusual Mass Spectrometric Dissociation Pathway of Protonated Isoquinoline-3-Carboxamides Due to Multiple Reversible Water Adduct Formation in the Gas Phase. *J Am Soc Mass Spectrom*.
14. Thevis, M.; Kohler, M.; Schlorer, N.; Schanzer, W. (2008) Gas phase reaction of substituted isoquinolines to carboxylic acids in ion trap and triple quadrupole mass spectrometers after electrospray ionization and collision-induced dissociation. *J Am Soc Mass Spectrom* 19, 151-158.
15. Fibrogen, *EP 1 538 160 A1*, in *European Patent Office*. 2005.
16. Ahlrichs; Bär, M.; Baron, H.-P.; Bauernschmitt, R.; Böcker, S.; Ehrig, M.; Eichkorn, K.; Elliott, S.; Furche, F.; Haase, F.; Häser, M.; Horn, H.; Huber, C.; Huniar, U.; Kattannek, M.; Kölmel, C.; Kollwitz, M.; May, K.; Ochsenfeld, C.; Öhm, H.; Schäfer, A.; Schneider, U.; Treutler, O.; von Arnim, M.; Weigend, F.; P., W.; Weiss, H. (2006) TURBOMOLE (Vers. 5.9). *Universität Karlsruhe*.