Untargeted detection of HIF stabilizers in doping samples: activity-based screening with a stable *in vitro* bioassay

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ABSTRACT: Hypoxia-inducible factor (HIF) stabilizers are listed in the World Anti-Doping Agency's prohibited list as they can increase aerobic exercise capacity. The rapid pace of emergence of highly structurally diverse HIF stabilizers could pose a risk to conventional structure-based methods in doping control to timely detect new investigational drugs. Therefore, we developed a strategy that is capable of detecting the presence of any HIF stabilizer, irrespective of its structure, by detecting biological activity. Previously developed cell-based HIF1/2 assays were optimized to a stable format and evaluated for their screening potential towards HIF stabilizers. Improved pharmacological characterization was established by the stable cell-based formats and broad specificity was demonstrated by pharmacologically characterizing a diverse set of HIF stabilizers (including enarodustat, IOX2, IOX4, MK-8617, JNJ-42041935). The methodological (in solvent) limit of detection of the optimal HIF1 stable bioassay towards detecting the reference compound roxadustat was 100 nM; increasing to 50 – 100 ng/mL (corresponding to 617 – 1233 nM in-well) in matching urine samples, owing to strong matrix effects. In a practical context, a urinary limit of detection of 1.15 μg/mL (95% detection rate) was determined, confirming the matrix-dependent detectability of roxadustat in urine. Pending optimization of a universal sample preparation strategy and/or a methodology to correct for the matrix effects, this untargeted approach may serve as a complementing method in anti-doping control, as theoretically it would be capable of detecting any unknown substance with HIF stabilizing activity.

INTRODUCTION

High altitude training is a well-known training method for athletes to increase their exercise performance as a result of an increased number of red blood cells to transport oxygen to the muscles.[1] The lower uptake of oxygen at high altitudes creates a state of hypoxia to which cellular processes adapt. Key factors for this cellular adaptation are the heterodimeric hypoxia inducible (transcription)factors (HIF). While the HIFβ-subunit is constitutively present in the cell, the α-subunit will be stabilized under hypoxia due to lower activity of prolyl hydroxylase domain enzymes (PHD), which initiate degradation of HIFα under normoxic conditions. Heterodimerization of stabilized HIFα with HIFβ yields a functional transcription factor, capable of regulating the expression of several genes, with downstream effectors counteracting the lower oxygen availability in the body (e.g. upregulation of erythropoietin, EPO, to increase the number of circulating red blood cells).[2]

While the response to hypoxia is a natural phenomenon, it can be and has been targeted pharmaceutically for therapeutic indications such as anemia (e.g. erythropoiesis stimulating agents, ESA).[3,4] Regarding anemic disorders, chemical stabilization of HIF through PHD inhibition has been one of the most widespread and advanced approaches of small-molecule based ESA therapies.[5] Being orally administrable, the therapeutic use of HIF stabilizers has added-value over injection with recombinant EPO. However, although HIF-stabilizers are emerging very quickly, most haven't been formally or globally approved and are currently being investigated in clinical trials.[6–11]

Given the ability of ESA therapies to increase oxygen supply, thereby possibly increasing aerobic exercise capacity, they appear on the World Anti-Doping Agency's (WADA) Prohibited list (S2.1). Together with methods for manipulation of blood and blood components they can be misused by athletes as *blood doping*, broadly defined by WADA as "any form of intravascular manipulation of the blood or blood components by

physical or chemical means".[12,13] In contrast to blood transfusions and misuse of EPO, HIF stabilizers should be directly detectable by sensitive, analytical detection methods such as mass spectrometry (MS) techniques.[14–20] However, while athletes can get their hands on these substances quite easily via internet suppliers (as has been the case for other performance enhancing substances)[21], the limited number of clinically approved HIF stabilizers makes it hard to obtain pure forms of these emerging drugs as reference standards. This poses a challenge for doping analysts: whereas a targeted screening for these substances implicitly relies on structural information on the active drug (and/or its metabolites), these data are often kept strictly confidential by pharmaceutical companies at the time of clinical trials. When the molecular structures of HIF stabilizing drug candidates are disclosed, multiple methods have to be developed and MS libraries have to be updated with the mass spectral characteristics as all these compounds show potential to be misused as doping agents in sports, even before pharmaceutical launch.[15,14,19,18,22] Noteworthy, High Resolution Mass Spectrometry (HRMS) can in principle be applied in the absence of mass spectral libraries or certified reference materials and, therefore, serve as a chromatographic- and MS-based untargeted screening method. However, HRMS methods can be time-consuming and expensive and the processing of untargeted acquisitions often still relies on targeted processing methods. In addition, unequivocal scoring of a positive result still requires a reference standard.

In addition to the delayed availability of structure and mass spectral characteristics, the high therapeutic interest in this class of ESAs also adds to the challenge for targeted techniques, as it results in a rapid pace of emergence of highly structurally diverse compounds. Preventive doping research therefore focused on assumed core scaffolds of investigational HIF stabilizers to timely develop analytical methods in order to comply with the explicit prohibition of these substances by WADA.[23] Tentative screening methods, developed based on information in patents and certain common structural motifs such as the glycineamide scaffold, have been suggested for the "development of non-targeted MS-based screening methods that should be able to detect whole compound classes, independent of the definite molecular structures of its individual members".[5,23] Similarly, a MS peculiarity of isoquinoline-derived HIF stabilizers was investigated, basing initial testing approaches on a neutral loss scan to detect mass differences specific to these compounds.[20] However, given that the class of (clinically proven functional) HIF stabilizers already comprises a large variety of structures, it can be envisaged that more compounds/structures/scaffolds are to come. Conventional 'structure-based' strategies may fail to detect these variants, as well as other drugs that -perhaps via another mechanism- lead to stabilization of HIFs. In order to avoid doping control lagging behind on the emergence of diverse investigational drugs, having at hand a strategy that would be capable of detecting the presence of any HIF stabilizer, irrespective of its structure or precise mechanism of action, would therefore be of great value.

Cell-based *in vitro* bioassays have shown potential as alternative untargeted approaches for the screening of structurally diverse (and/or designer) molecules in other doping classes involving anabolic agents.[12,24,25] These 'steroid' bioassays exploit the natural biology of androgenic signaling in a cell on which the anabolic doping agents act to mediate their major effects in humans.[26,27] Similarly, we recently developed cell-based activity-based HIF bioassays exploiting the natural phenomenon of oxygen sensing in which HIF signaling, and more specifically HIF stabilization, is involved.[28] These bioassays have already shown broad specificity, capable of generating concentration-response curves for multiple recently approved and current investigational HIF stabilizers and other HIF-related drugs.[28,29] In the current study, we optimized the assay format, generating stable cell systems, and evaluated the capability of this untargeted activity-based approach to detect HIF stabilizers in biological samples within a doping control context.

MATERIALS & METHODS

Retroviral Constructs

The development and selection of the optimal combination of HIF1/2α- and HIF1β-constructs in the NanoBiT® system was described previously.[28] The resulting coding sequences of the SmBiT-HA-HIF1α, SmBiT-HA-HIF2α and LgBiT−FLAG-HIF1β constructs were cloned into retroviral vectors pLZRS-IRES-dNGFR (SmBiT-HA-HIF1α and SmBiT-HA-HIF2α) or pLZRS-IRES-EGFP (LgBiT−FLAG-HIF1β) (details in Supporting Information 1). The integrity of all retroviral plasmids was verified by Sanger sequencing (Eurofins Genomics LLC, Ebersberg). Insertion into these retroviral vectors ensured co-expression of specific markers; either truncated nerve growth factor receptor (dNGFR) for the HIFα-constructs or enhanced green fluorescent protein (EGFP) for the HIF1β-construct, allowing flow cytometry-assisted cell sorting (FACS) and routine verification of the stability of the cell lines via flow cytometry.

Stable cell line generation

Aiming at reducing the workload and variability associated with transient transfection, retroviral transduction was used to generate two stable cell systems, expressing either SmBiT-HA-HIF1α or SmBiT-HA-HIF2α in combination with LgBiT−FLAG-HIF1β. Transduction was according to previously described protocols (details in Supporting Information 1).[30] Cells highly expressing both the HIF1/2α fusion protein (measured by dNGFR surface expression) and the HIF1β fusion protein (measured by EGFP fluorescence) were selected via FACS on a BD FACS Fusion, equipped with 405, 488, 561, and 640 nm lasers (BD Biosciences, Erembodegem, Belgium). For the detection of HIF1/2α-linked expression of dNGFR, an APC-labeled antibody against dNGFR was used (Chromaprobe Inc., MO, US).

Cell Culture

All HEK 293T cell lines (original HEK 293T and stably transduced HIF1 and HIF2 cell lines) were routinely maintained at 37 °C, 5% CO_2, and under humidified atmosphere and were passaged at 80−90% confluence. They were cultured in Dulbecco's Modified Eagle Medium (DMEM, GlutaMAX™ containing high glucose levels) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/ mL amphotericin B. Stability of the generated cell lines was monitored by flow cytometric analysis of the co-expressed markers.

HIF heterodimerization bioassays

Stable or transfected cells (transfection protocol was previously described)[28] were seeded into the inner wells of Poly-D-Lysine coated white 96-well plates at a density of 5×10^4 cells/well. The wells at the outside of the plate were at all times repleted with 200 µL phosphate-buffered saline (PBS incl. calcium & magnesium, Gibco). After overnight incubation the cells were washed twice with OptiMEM I™ Reduced Serum Medium and treated for 24 hours with 10 µL 10× reference stock solutions (1:10 dilution in well) or 100µL reconstituted sample extract. Solvent concentrations on the cells were limited to maximally 0.1% DMSO. The assay was performed by adding 25 µL of the Nano-Glo Live Cell reagent, which was prepared by diluting the Nano-Glo Live Cell substrate 1:20 in Nano-Glo LCS dilution buffer (commercially available from Promega, Madison, WI, USA). This nonlytic detection reagent contains the cell-permeable furimazine substrate. Subsequently, luminescence was immediately monitored for 120 minutes in a TriStar^2 LB 924 multimode microplate reader (Berthold Technologies GmbH & Co., Germany).

Data analysis & Statistical analysis

Data were obtained in a minimum of three independent experiments and data points were measured in duplicate. Luminescence (relative light units, RLU) was plotted over time, generating time-heterodimerization profiles. The area under the curve (AUC) over a period of 120 minutes (incl. subtraction of the blank

AUC) served as a measure for HIF heterodimerization. Increased signal compared to blank showed the occurrence of HIF stabilization upon treatment with reference stock solutions. Further data analysis and statistical analysis was performed using GraphPad Prism 9 software (San Diego, CA, USA). Concentration-response curves of reference standards were fitted via non-linear regression (four-parametrical logistic fit) to derive potency (EC_{50}) and efficacy (E_{max}) values. The E_{max} was expressed as a percentage relative to the maximal effect of the PHD inhibitor roxadustat (FG-4592, E_{max} arbitrarily set at 100%). Statistical analysis includes the non-parametric Kruskal-Wallis test including a Dunn's correction to account for multiple comparisons.

Urine sample preparation

Urine samples were spiked with specific concentrations of HIF stabilizer for different experiments (maximum 1% spiking volume to original matrix volume). Spiked and non-spiked urine samples were prepared for bioassay analysis through liquid-liquid extraction of 1 mL urine. The samples were acidified with 500 μL acetate buffer (pH 4.5) upon vortexing and were extracted with 3 mL *tert*-butyl methyl ether. Following thorough shaking and centrifugation (2500 rpm, 10 min), the organic phase was evaporated to dryness under a nitrogen stream at 40°C. Sample extracts were reconstituted in 230 μL 0.1% DMSO/OptiMEM.

RESULTS & DISCUSSION

Stable expression of the HIF bioassay

The HIF bioassays utilize a functional complementation-based approach to monitor protein interactions within living cells by making use of the NanoLuc Binary technology (NanoBiT).[28,31] By coupling the two inactive subunits of the split-nanoluciferase (NanoLuc), LgBiT (18 kDa) and SmBiT (1 kDa), to the individual subunits of the HIF transcription factor, HIF1α/2α on the one hand and HIF1β on the other, this technology enables direct and real-time monitoring of HIF1 and HIF2 heterodimerization. This natural protein interaction brings the coupled NanoLuc-subunits into close proximity, allowing restoration of nanoluciferase activity as a direct effect of HIF heterodimerization. HIFα stabilization by means of doping agents implicitly results in an increased interaction with HIF1β, yielding an increase in luminescence, which can be measured in the bioassay.

We previously set up and applied these HIF bioassays in a transient format, demonstrating applicability for both clinical PHD inhibitors (i.e. HIF stabilizers) and general hypoxia mimetics.[28] In this study, we report on the establishment of two HEK293T cell lines, stably expressing both heterodimerization assay fusion proteins (either SmBiT-HA-HIF1α or SmBiT-HA-HIF2α and LgBiT−FLAG-HIF1β). After retroviral transduction, these stable cell lines were sorted via FACS and further monitored for the percentage of expressing cells and the level of expression, based on the corresponding co-expressed markers (dNGFR for HIFα constructs, EGFP for the HIF1β construct). These markers are co-expressed from the same mRNA, as the transduced constructs are bicistronic. Subsequent sorting cycles were applied, with functional verification in between, until optimal sensitivity was reached, resulting in three sorting cycles for the HIF1 stable cell line and four cycles for the HIF2 stable cell line. So far, the number of double positive cells and the level of expression has remained stable, but repeated sorting rounds are possible should there be a need. As it is possible that the overexpression of the HIF subunits could impose a negative effect on growth, which would jeopardize the cell line's utility in long-term, cells were only utilized until passage 25 for experiments. However, visual monitoring of the cell cultures suggests normal growth.

Improved pharmacological characterization

To comparatively evaluate transient and stable HIF1 and HIF2 bioassays, we generated concentration-response curves for three HIF stabilizers (roxadustat, used as a reference, daprodustat and vadadustat), to derive potency (EC_{50}) and efficacy (E_{max}) values (Figure 1, Table 1). Previously, no EC_{50} value could be derived for vadadustat with the HIF1 transient assay.[28] Here, a potency of 12.2 μM could be estimated, although the

span of the 95%-confidence interval was still large (Table 1). As previously observed, the HIF2 transient bioassay yielded a high uncertainty for the EC_{50} and E_{max} values for daprodustat and vadadustat.[28] Although for daprodustat a plateau (average E_{max} of 55.0%) was reached when considering individual experiments, the large variation in the transient format led the software to incorrectly extrapolate the sigmoidal curve, so that it seemingly does not reach a plateau. Hence, no E_{max} could be fitted and therefore no EC_{50} value could be derived. For vadadustat no (complete) confidence intervals could be estimated due to the high variability in the (non-optimized) format.

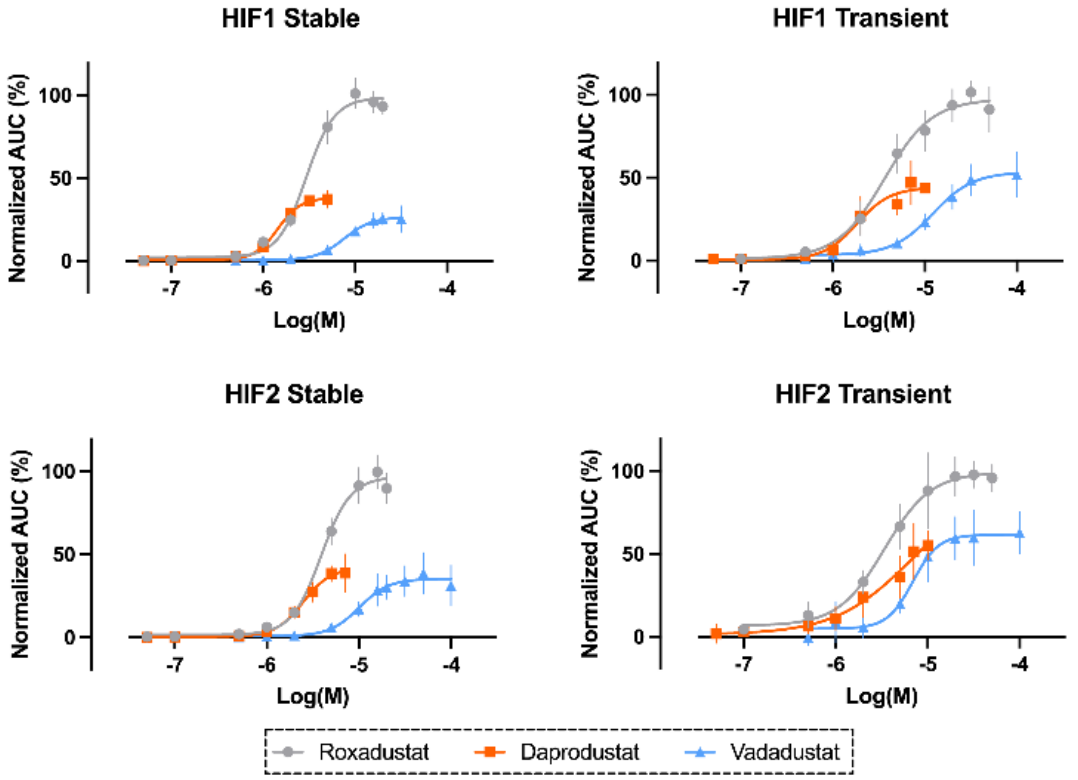

Figure 1. Concentration-response curves from side-by-side pharmacological characterization in stable and transient assay format for HIF1 and HIF2 stabilization. AUC values were normalized experiment-individually to the E_{max} of roxadustat. Data are represented by the mean ± standard error of the mean (SEM).

Table 1. Potency (EC_{50}) and efficacy (E_{max}) values from side-by-side pharmacological characterization in stable and transient assay format, accompanied by the corresponding 95%-confidence intervals (CI) between brackets.

Compound	HIF1 Stable		HIF1 Transient		HIF2 Stable		HIF2 Transient	
	EC_{50} (µM)	E_{max} (%)	EC_{50} (µM)	E_{max} (%)	EC_{50} (µM)	E_{max} (%)	EC_{50} (µM)	E_{max} (%)
Roxadustat	2.93 [2.42-3.58]	98.4 [91.1-107]	3.64 [2.01-6.36]	97.4 [84.6-130]	3.89 [3.06-4.87]	96.7 [88.0-110]	3.39 [1.53-8.12]	99.2 [85.2-158]
Daprodustat	1.44 [1.19-1.75]	38 [33.6-44.4]	1.84 >1.06	44 >34.3	2.47 [1.87-5.83]	41.9 [34.0-79.3]	/[b]	>40.2 [a]
Vadadustat	7.57 [4.78-31.2]	26.4 [21.3-62.8]	12.2 [5.83-380]	53.4 [41.2-178]	10.1 [5.90-17.1]	35.3 [28.2-50.2]	6.90 /	61.6 >47.9

[a]No specific fitted Emax is reported as the fit was incorrectly extrapolated by the software
[b]No EC_{50} is reported as the fit was incorrectly extrapolated by the software

Despite the large variability with the transient assay format, the results over time seem rather consistent.[28] Firstly, the potency of daprodustat (EC_{50}=1.84 µM) for HIF1 stabilization is in essence the same as the one previously derived (1.86 µM).[28] Secondly, while the derived EC_{50} of roxadustat is now somewhat lower (HIF1 EC_{50}=3.64 µM, HIF2 EC_{50}=3.89 µM) compared to our previously published results with the same transient

format (HIF1 9.53 µM, HIF2 5.50 µM), the ranking order has remained the same, with increasing potency from vadadustat < roxadustat < daprodustat.[28] Highly similar relative E_{max} values over time also resulted in the same ranking order as compared to our previous study: daprodustat (HIF1, 44% *versus* 37.9%; HIF2, 55.0% *versus* 51.8%) < vadadustat (HIF1, 53.4% *versus* 53.5% at 200µM; HIF2, 61.6% *versus* 60.9%) < roxadustat (used as a reference for normalization).[28]

Overall, the results in this study from the stable HIF bioassays matched those of the transient assay formats, with two important differences. Firstly, the ranking order for efficacy has changed, as vadadustat was less efficacious in the stable format, resulting in lower E_{max} values compared to daprodustat for both HIF1 and HIF2 heterodimerization (vadadustat HIF1 E_{max}=26.4%, HIF2 E_{max}=35.3%; daprodustat HIF1 E_{max}=38%, HIF2 E_{max}=41.9%). Secondly, the stable HIF bioassays outperformed the transient assay format in terms of variability as in every instance complete 95% confidence intervals could be obtained, additionally covering smaller spans when compared to those generated in the transient formats, allowing reliable determination of potency and efficacy.

Broad specificity

Based on the analysis of reference standards, we previously showed that these bioassays can also detect general hypoxia mimetics (e.g., $CoCl_2$, iron chelator desferrioxamine, and general proteasome inhibitor MG-132).[28,29] Given the continuous emergence of new HIF stabilizers in medical research, which can all be considered as potential doping agents, broad specificity is of high importance for this untargeted detection method. We therefore used the newly developed stable HIF bioassays to pharmacologically evaluate newer or less clinically evaluated PHD inhibitors to demonstrate the potential of these bioassays to further expand the list of compounds with presumed HIF stabilizing capacity that may be detected based on the activity-based principle (Figure 2).

The highly similar EC_{50} values for roxadustat in Tables 1 and 2, although different cell freezing batches were used for these consequential experiments, support the robustness of the stable bioassays. Relative to roxadustat, enarodustat was a less potent and less efficacious HIF stabilizer (EC_{50}: HIF1 11.3 µM, HIF2 18.7 µM; E_{max} HIF1 73.2%, HIF2 74.5%) (Table 2). IOX2 (readily detected in early 2020 in a racehorse doping control sample)[16,32] showed less HIF stabilizing activity compared to roxadustat and enarodustat. Comparing HIF1/2 isoforms, a higher efficacy (HIF1 E_{max}=40.5%, HIF2 E_{max}=61.2%) but lower potency (HIF1 EC_{50}=5.93 µM, HIF2 EC_{50}=8.48 µM) for HIF2 stabilization was observed, although confidence intervals are overlapping (Table 2). JNJ-42041935 was almost equipotent (HIF1 EC_{50}=3.55 µM, HIF2 EC_{50}=4.24 µM) with roxadustat, with highly overlapping confidence intervals (Table 2).[33] However, it had a much lower efficacy (HIF1 E_{max}=33.2%, HIF2 E_{max}=30.5%). On the other hand, MK-8617, being the least efficacious compound regarding HIF stabilization of both HIF1 (E_{max}=15.7%) and HIF2 (E_{max}=18.3%), was found to be the most potent HIF stabilizer (HIF1 EC_{50}= 0.203 uM, HIF2 EC_{50}=0.301 uM). The second most potent HIF stabilizer characterized so far is IOX4 (HIF1 EC_{50}=0.731 uM, HIF2 EC_{50}=1.30 uM), with efficacies for HIF1 (E_{max}=35.0%) and HIF2 (E_{max}=37.7%) stabilization similar to those of JNJ-42041935.

For most of these PHD inhibitors, MS-based methods have been developed and drug metabolism has been investigated both in the context of preventive doping research since 2012, as in reaction to various adverse analytical findings since 2015 for several HIF stabilizers.[5,16,17,19,34] However, for these MS-based routine initial testing methods there is the need of continuous updating of MS-libraries to detect analytes that represent newly emerging drug candidates. In contrast, this study serves as proof-of-concept that the presence of any HIF stabilizing compound may be detectable by this untargeted *in vitro* bioassay. This demonstrates a future-proof screening principle in case of further emergence of new drug candidates, deemed as potential doping agents, without the need of updating the method.

Potential of the bioassay formats for screening purposes

To evaluate the potential of these bioassays for screening purposes in doping control, we first evaluated the intrinsic quality of the stable and transient assay formats by determining and comparing the statistical Z'-factor. This factor is a characteristic parameter for the quality of assays, based on the assay signal dynamic range and data variation on positive and negative controls, favoring assays for screening purposes with a factor > 0.5.[35]

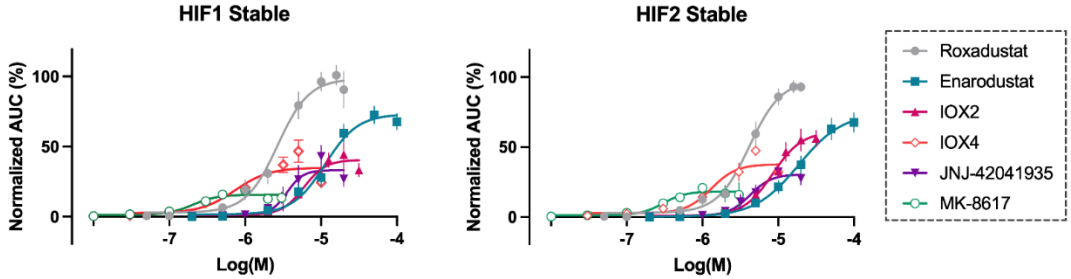

Figure 2. Concentration-response curves of selected PHD inhibitors in the optimized stable HIF1 and HIF2 bioassay. AUC values were normalized experiment-individually to the E_{max} of roxadustat. Data are represented by the mean ± standard error of the mean (SEM).

Table 2. Potency (EC_{50}) and efficacy (E_{max}) values from pharmacological characterization in stable assay format for HIF1 and HIF2 stabilization, accompanied by the corresponding 95%-confidence intervals (CI) between brackets.

Compound	HIF1 Stable		HIF2 Stable	
	EC_{50} (µM)	E_{max} (%)	EC_{50} (µM)	E_{max} (%)
Roxadustat	2.66 [1.85-3.81]	98.42 [87.8-117]	4.14 [3.30-5.43]	97.9 [88.6-116]
Enarodustat	11.3 [8.51-15.4]	73.2 [65.1-85.8]	18.7 [12.7-52.0]	74.5 [62.4-119]
IOX2	5.93 [3.28-9.25]	40.5 [32.2-63.3]	8.48 [6.17-15.1]	61.2 [51.0-92.0]
IOX4	0.731 [0.147-1.55]	35.0 [28.1-51.6]	1.30 [0.766-2.16]	37.7 [31.7-49.0]
JNJ-42041935	3.55 [2.03-5.32]	33.2 [24.7-43.7]	4.24 [2.93-6.03]	30.5 [25.2-39.3]
MK-8617	0.203 [0.0224-0.401]	15.7 [12.2-23.7]	0.301 [0.128-0.492]	18.3 [14.8-25.4]

A negative Z'-factor implies that screening would essentially be impossible.[35] We could conclude that the decreased variability with the stable format (HIF1 Stable Z' = 0.68 ± 0.19, HIF2 Stable Z' = 0.35 ± 0.45) benefits the assay's quality compared to the transient format (HIF1 Transient Z' = -0.19 ± 0.21, HIF2 Transient Z' = -0.72 ± 0.13). Secondly, within the same format (transient or stable) the HIF1 bioassay is more qualified for screening purposes than the HIF2 bioassay. In fact, in two out of three independent experiments, the HIF1 stable bioassay showed excellent quality with a Z'-factor > 0.8 (Supporting Information 2, Figure S1).

Next, the sensitivity of each assay format was determined for a concentration range of the HIF stabilizer roxadustat, used as a reference compound in this study. Fold changes of the heterodimerization signals (S) to the mean of the blanks (B_{mean}) were calculated to determine the lowest concentration that could be distinguished from solvent control (Figure 3). Because of the decreased variability with the stable bioassays, the sensitivity increased. Both the HIF1 and HIF2 transient assays were capable to significantly ($p \leq 0.01$) distinguish 1 µM of roxadustat from solvent control. For HIF2 this is in line with our previous results, whereas

the HIF1 bioassay in a transient format was previously capable of detecting down to 100 nM of roxadustat.[28] This discrepancy may be accounted for by variability in the transfection efficiency over time and the use of new DNA stock solutions. With a stable format, both HIF bioassays can significantly distinguish 10-fold lower concentrations of roxadustat (HIF1 $p \leq 0.0001$, HIF2 $p \leq 0.001$). Dunn's multiple comparison test showed (marginally) non-significant results for the detection of 50 nM roxadustat with the HIF1 (p=0.0535) and HIF2 (p=0.0857) stable formats.

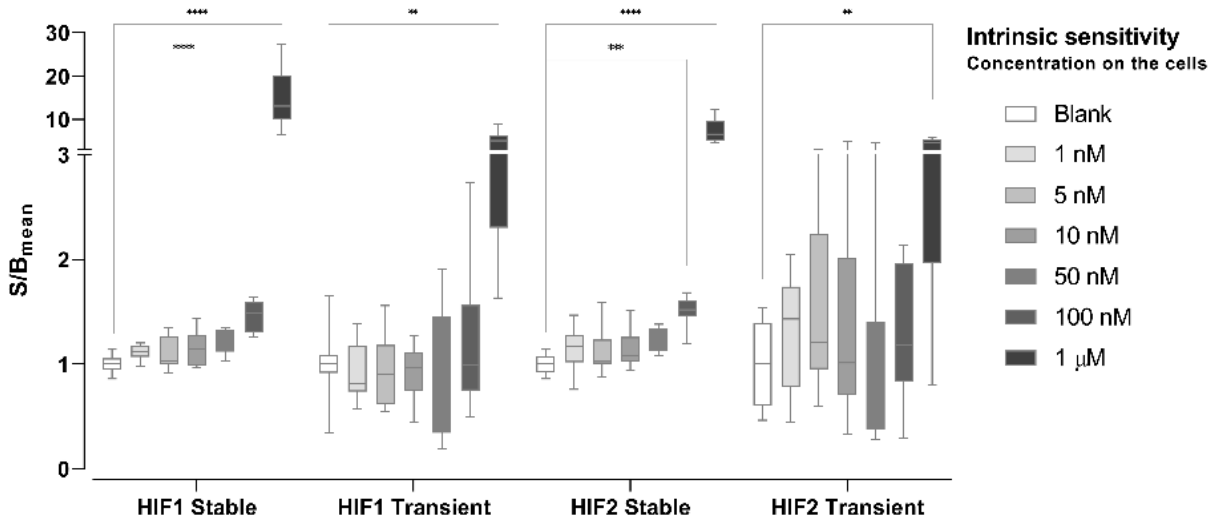

Figure 3. Sensitivity of the bioassay towards roxadustat in solvent. Comparison of the signal-to-blank ratios of different molar concentrations of roxadustat in assay medium using the stable and transient assay formats of the HIF1 and HIF2 bioassays. Data are represented as boxplots (n=10) with 2.5-97.5 percentile whiskers. Statistical analysis: Kruskal-Wallis test with Dunn's multiple comparisons, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

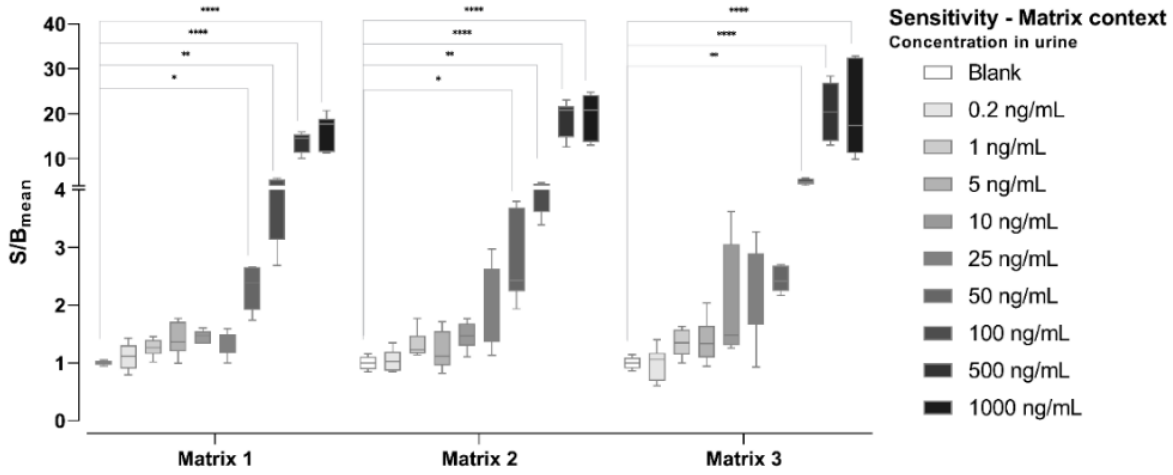

Figure 4. Sensitivity of the bioassay towards roxadustat in urine. Signal-to-blank ratios of the stable HIF1 bioassay for the detection of (spiked) roxadustat concentrations in independent urine matrices (1-3). Data are represented as boxplots (n=6) with 2.5-97.5 percentile whiskers, including urine concentrations (before extraction). Statistical analysis: Kruskal-Wallis test with Dunn's multiple comparisons, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Based on the evaluation of the intrinsic screening potential (Z'-factor) and the methodological limit of detection of roxadustat in solvent, the HIF1 bioassay in a stable format was chosen to move forward for further testing on biological matrices. In addition, the choice for a stable cell line practically implies a lower workload and lower variability in experimental results, making it more cost-efficient from a routine analysis point of view.

Activity-based detection of roxadustat in urine samples

Although elsewhere and here we demonstrated the bioassays to be capable of detecting a wide variety of HIF stabilizers as reference standards, it remained to be evaluated whether this principle would also work in biological matrices.[28,29] Indeed, in order for the HIF1 bioassay to be applicable to anti-doping testing, it should be capable of detecting HIF (stabilizing) activity in biological samples, preferably urine. To the best of our knowledge, no endogenous compounds have been reported in urine that could interfere with the activity-based read-out (i.e. would lead to HIF heterodimerization). This is an important difference with androgenic bioassays, that have to cope with endogenous bioactivity due to the presence of endogenous androgens in urine such as testosterone.[25,26] Given the aim for a universal procedure, a straightforward sample preparation was applied on the urine samples, including acidification and liquid-liquid extraction with *tert*-butyl methyl ether, similar to the confirmation procedure of a positive case of roxadustat as reported by Buisson & Marchand *et al*.[17] Recovery was analytically investigated for a panel of HIF stabilizers with varying structures (daprodustat, desidustat, enarodustat, IOX4 and JNJ-42041935) and ranged from 85-97% (CV 3-18%) at a spiked concentration of 10 ng/mL and from 81-96% (CV 3-8%) at a spiked concentration of 500 ng/mL, with no pronounced differences between different HIF stabilizers (Supporting Information 3, Figure S2).

As the detectability of compounds and the sensitivity of the bioassay can be influenced by the matrix, the sensitivity of the HIF1 stable bioassay was determined for the detection of roxadustat in spiked urine. Extracts from three independent urine matrices that had been spiked with a concentration range of roxadustat (0.2, 1, 5, 10, 25, 50, 100, 500 and 1000 ng/mL) were subjected to the HIF1 stable bioassay. Fold changes of the heterodimerization signals (S) to the corresponding blank (non-spiked urine matrix, B_{mean}) were calculated to determine the lowest concentration in each urine matrix that could be distinguished from the corresponding blank signal (Figure 4). Whereas the sensitivity to detect roxadustat in solvent was determined to be 100nM (i.e., in-well concentration, see above), the sensitivity to detect roxadustat in urine ranged from 50-100 ng/mL urine (corresponding to 617 – 1233 nM in-well, after sample extract reconstitution; the calculation for conversion of urinary concentrations to concentrations in-well can be found in Supporting Information 4). Importantly, different urine matrices resulted in different minimum concentrations of roxadustat that could be detected, all ensuing a lower sensitivity than when a matrix-free reference standard was tested. Of note, we found the sensitivity to correlate with the baseline signals obtained for the blank (non-spiked) urine samples (Supporting Information 5, Figure S3): the decreasing baseline signals observed for urine matrices 1 to 3 in Figure 4 coincided with a decreased sensitivity, from 50 nM (matrices 1 & 2) to 100 nM (matrix 3). This indicates signal suppression due to the presence of matrix.

The underlying cause for this matrix effect is likely related to multiple contributing factors. Evaluation of 10 independent urine matrices yielded significantly different baseline-luminescence values (Friedman test, p= 0.0181). Readily when considering EGFP positivity (EGFP being co-expressed with SmBiT-HIF1α and considered as a surrogate measurement for the number of cells – and hence cell viability), significant differences (Friedman test, p= 0.0108) were found (Supporting Information 6, Figure S4). However, the observed differences in EGFP fluorescence and the differences in bioassay luminescence were not of the same order of magnitude. Hence, there are other factors influencing the bioassay read-out, which can be related to the urine composition: creatinine levels, the specific gravity and the pH of the samples, the conductivity, the osmolality, the amount of uric acid and the amount of iodine in urine were all (partially) negatively correlated to the bioassay read-out (Supporting Information 6, Figure S5).

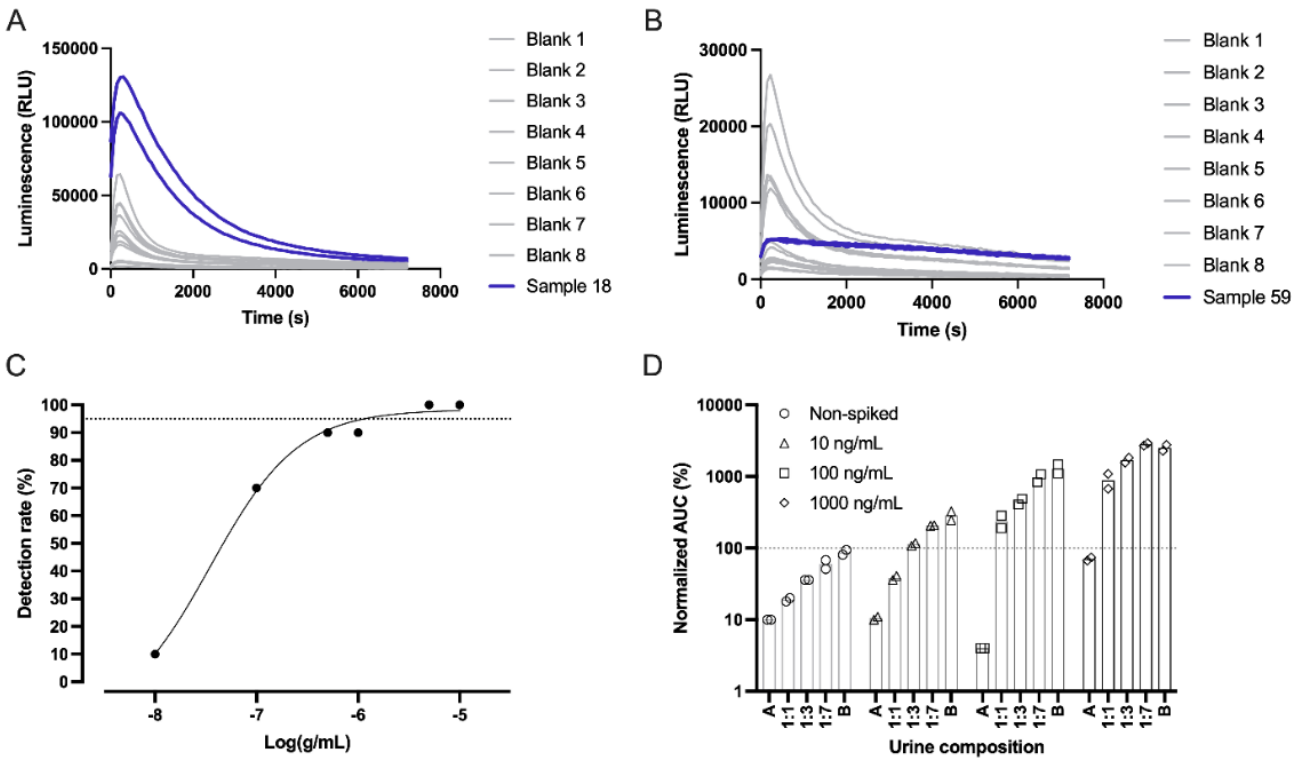

Figure 5. Determination and understanding of a practical limit of detection (LOD) for untargeted screening with the HIF1 stable bioassay. A,B) Representative examples of positive results, based on higher peak luminescence (A) or on a sustained heterodimerization profile (B). Data are represented as duplicate measurements. C) Sigmoidal fit of the detection rate of spiked urine samples with roxadustat. The determined LOD is the concentration that results in a detection rate of 95% (dotted line). D) Cumulative luminescence in the bioassay of extracts from concentrated urine matrix A, less concentrated urine matrix B and mixtures of both (e.g., 1:1 mixture of A and B) at different spiked concentrations of roxadustat. Data are normalized to the mean area under the curve (AUC) of solvent controls (0.1% DMSO/OptiMEM) and are represented on a log scale

In general, more concentrated urine samples (with high values for parameters such as creatinine, S.G., osmolality *etc.*) yielded lower baseline signals relative to solvent control, indicative of signal suppression (e.g. urine A in Figure 5D, urine C in Supporting Figure S7), whereas less concentrated urine matrices resulted in little to no signal suppression (e.g. urine B in Figure 5B, urine D in Supporting Figure S7). However, the baseline signals varied substantially between independent experiments and, correspondingly, not in every independent experiment a significant correlation was observed (urine parameter *versus* baseline signal) (Supporting Information 6, Figure S5).

To cope with this variability in urine-dependent baseline signals and evaluate a realistic anti-doping testing scenario, we determined a practical limit-of-detection of the stable HIF1 bioassay. For this, we used eight blank urine matrices, encompassing a high variability (pH: 5.0-8.0, S.G.: 1.006-1.027), as negative controls. Ten other urine matrices (pH: 5.0-8.0, S.G.: 1.006-1.025) were spiked with roxadustat to obtain 7 concentration levels (non-spiked, 10 ng/mL, 100 ng/mL, 500 ng/mL, 1 μg/mL, 5 μg/mL, 10 μg/mL). These samples were randomized and blind-coded for analysis with the HIF1 stable bioassay. In every experiment, blind-coded samples were run alongside the 8 various blanks, a positive control (10 μM roxadustat in solvent) and a negative solvent control (0.1% DMSO/OptiMEM). Scoring of the samples (positive/negative) was performed by visual comparison of the sample heterodimerization profile with the broad range of blank profiles. Two visual indications were considered to score a blind-coded sample as positive: i) higher peak compared to all blanks (example in Figure 5A), ii) sustained heterodimerization profile within the range of the blanks (example in Figure 4B). Based on these criteria no false positives were detected and a detection rate of 100% was obtained for samples spiked at 5 or 10 μg/mL. One sample out of ten containing 10 ng/mL

roxadustat could be detected positive. In the least concentrated urine matrix in the panel (S.G.=1.006), every spiking level was detected positive (Supporting Information 7, Figure S6, urine matrix A). The detection rates for 100 ng/mL, 500 ng/mL and 1 μg/mL were 7/10, 9/10 and 9/10, respectively, including positive results for urine matrices with various densities (*cfr*. Supporting Information 7, Figure S6 for the corresponding urine matrices). Based on a three-parameter sigmoidal fit of the detection rates, a limit of detection (LOD), corresponding with a detection rate of 95%, was determined to be 1.15 μg/mL (Figure 5C). The fact that this LOD is higher than the one in solvent or when considering matched (spiked-non-spiked) urine samples can be attributed to the above-described matrix effects. Indeed, also in these samples we observed that less concentrated urine matrices result in less signal suppression compared to solvent controls, leading to easier detection of spiked HIF stabilizer concentrations. We experimentally confirmed this observation by generating mixtures of concentrated and less concentrated independent urine matrices (representative experiments in Figure 5B & Supporting Information 8, Figure S7). The more concentrated a mixture, the higher the spiked concentration of roxadustat needed to be before the signal rose above solvent control. These results suggest that there may be an opportunity -whether in the bioassay itself or in the sample preparation- to cope with the true underlying cause(s) and correct for this urine-dependent effect. However, more research is needed to come up with a solution that alleviates the matrix dependency of the current set-up. Efforts have been made based on specific gravity and creatinine levels, but no adequate correction method was identified up to date.

Untargeted detection of HIF stabilizers: current status & future perspectives

Atkinson *et al*. stated in 2020, that "athletes and their trainers are frequently ahead of the curve when it comes to exploiting the science of performance enhancement and evading detection, and this trend seems unlikely to change".[13] Untargeted detection of HIF stabilizers via detecting biological activity may have the potential to serve as a complementary tool in doping control, to help to cope with the continuous emergence of new HIF stabilizing drug candidates with varying structures. This study serves as proof-of-concept for activity-based testing of HIF stabilizers in urine samples, with several potential (research & doping control) applications such as retrospective studies to assess the effectiveness of preventive doping analyses (is doping control 'ahead of the game' or 'lagging behind'?), guidance of intelligent testing strategies and (maybe in the future) untargeted screening of doping control samples.

Currently, the determined LOD for roxadustat is still (much) higher than the regular WADA requirements for analytical testing (required detection level of 2 ng/mL).[18,36] These required detection levels have to be met by analytical methods in doping control to reliably identify and confirm adverse analytical findings (AAFs). Due to the currently high LOD that was observed when evaluating urine matrices, the sensitivity of the studied cell-based approach is at this point not sufficient to serve as an alternative screening procedure for biofluids. Nevertheless, the newly developed untargeted activity-based approach could be of added-value in early detection of newly emerging HIF stabilizers prior to their inclusion in routine analytical methods, as they are being used at high levels due to a (presumed) lack of detectability. E.g., a threshold plasma concentration of 4.1 μg/mL was reported for roxadustat to result in pharmacological increases in EPO levels, suggesting a need for high blood levels to obtain performance enhancing effects.[37] In line with this, Buisson & Marchand *et al*. reported on a positive case of roxadustat, where a concentration of 18 μg/mL was measured, which is far above our current LOD.[17] Importantly, the LOD that was determined here for the detection of roxadustat in urine, will differ amongst HIF stabilizers because of different intrinsic pharmacology. More potent HIF stabilizers (e.g., MK-8617 or IOX4, as determined by pharmacological characterization, see above) will likely result in lower limits of detection, whereas less potent HIF stabilizers (e.g., enarodustat) might result in higher LODs. Accordingly, less potent HIF stabilizers will likely be taken at higher doses, due to their lower capability of enhancing performance – hence, typically yielding higher concentrations. However, that is the subject of other research in anti-doping.

For the bioassay to serve as an untargeted future-proof screening tool, a universal methodology is required. Accordingly, both the detection principle and the sample preparation method should pursue universality. This study establishes broad specificity for activity-based detection of HIF stabilizers, including their detection in biofluids. The aim for a consonant universal sample preparation method, led to the implementation of a straightforward (acidified) liquid-liquid extraction in this study. Other sample preparations have already been explored (consequential basic-acidic LLE, supported liquid extraction, solid-phase extraction with C18, HLB and anion-exchange columns), but led to inferior results for matrix effects and/or overall recovery. Whereas it is possible to develop a dedicated sample preparation procedure for roxadustat to enhance sample clean-up and match the superior results we obtained in solvent, this would contrast our primordial aim of having a fully universal method for all HIF stabilizers, including highly variable structures. Alternative roads to cope with the matrix effects were taken based on the insights that were obtained on the phenomenon, including correction methods for urine composition (e.g., specific gravity, creatinine) and different assay protocols. However, at this point none of the changes to the protocol showed superior results to the current set-up.

While we acknowledge the limitations of the current sensitivity of our approach, the universal method could already be useful at its current performance level to identify suspicious samples and store these for retesting (in a period of up to ten years), as allowed under the current anti-doping regulations. Structural knowledge of new HIF activating compounds and their metabolites can lead to more optimized MS-based methods, which may identify currently unknown HIF-related compounds upon re-analysis. Hence, the activity-based method can serve as an auxiliary detection method for MS-based methods. It can raise awareness and caution for samples containing newly emerging HIF stabilizers prior to analytical method development and routine implementation in doping control of analytical testing methods that will sensitively identify and confirm AAFs. In time, a better compromise or additional sample pretreatment (such as sample dilution) might be explored to further combine optimized universal sample preparation with a better sample clean-up or improved compatibility with the biological read-out. This could eventually reduce matrix effects, further improve the LOD and pave the way for a potential use of our bioassay as an alternative first-line screening tool.

An important future perspective in studying activity-based detection of HIF stabilizers is to evaluate the biological activity of excreted metabolites. As is the case with almost all xenobiotics, HIF stabilizers are (or can be) metabolized in the human body to derive more polar phase I or phase II metabolites. This has already been demonstrated for molidustat, which is excreted in urine almost exclusively as its glucuronidated metabolite, which can be overcome by including a deglucuronidation step in sample preparation methods.[38] For several HIF stabilizers (molidustat, roxadustat, daprodustat, desidustat, vadadustat) and other drugs impacting HIF, metabolites (human and/or equine) have been studied in the context of doping control.[14,15,34,39,40] The applied bioassay measures *combined HIF stabilizing activity,* meaning that the activity of every compound (parent drug & active metabolites) is relevant when screening biofluids with the assay, which can be considered an advantage. When a parent drug is heavily metabolized, as is the case for e.g., daprodustat, the activity of metabolites could increase the bioassay's sensitivity (as has been shown for a similar activity-based method detecting synthetic cannabinoids).[41] On the other hand, a lack of active metabolites and little excretion of the parent compound can hamper activity-based detection overall. Research on the remaining (HIF stabilizing) activity of individual metabolites in the form of reference standards is needed to get insight into that aspect that is also relevant for the practical applicability of activity-based screening methods.

Currently approved methods for indirect detection of doping include the athlete biological passport (ABP). More specifically, the blood module of the ABP is capable of detecting the administration of EPO through changes in reticulocyte counts and hematocrit.[13] Whether it would be capable of detecting the use of HIF stabilizers is currently unknown. In the case of Buisson & Marchand *et al*. changes in the abnormal blood profile score (ABPS, composing of hematocrit, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration) were observed, but they were not sufficient to alarm the expert panel for review.[17]

CONCLUSION

This study provides a proof-of-concept for activity-based detection of HIF stabilizers as an untargeted screening approach. Untargeted detection of performance enhancing substances can aid doping control to be proactive, where athletes don't seem to hold back in exploiting scientific advances that could enhance performance. Currently, applicability of the improved HIF1 bioassay in a doping control context is still hindered by matrix effects negatively impacting the sensitivity. While LC-MS methods are not dependent on pharmacological activity and provide excellent sensitivity, activity-based methods do not rely on molecular structures and prior knowledge thereof. Therefore, activity-based detection could complement to the qualities of chromatography and MS and aid in the (preliminary) detection of unknown substances.

ASSOCIATED CONTENT

Supporting Information

Additional experimental details, methods and results, including:
Supporting Information 1: Detailed materials & methods
Supporting Information 2: Detailed results on Z'-factor
Supporting Information 3: Analytical evaluation of sample preparation recovery
Supporting Information 4: Conversion of urinary concentrations to concentrations in-well
Supporting Information 5: Baseline signals of independent urine matrices
Supporting Information 6: Investigations of matrix effect
Supporting Information 7: Scoring of blind-coded samples for the LOD determination
Supporting Information 8: Effect of urine composition: second representative experiment

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