High-Throughput Fluorescence-Based Activity Assay for Arginase-1

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Abstract
Arginase-1, which converts the amino acid L-arginine into L-ornithine and urea, is a promising new drug target for cancer immunotherapy, as it has a role in the regulation of T-cell immunity in the tumor microenvironment. To enable the discovery of small-molecule Arginase-1 inhibitors by high-throughput screening, we developed a novel homogeneous (mix-and-measure) fluorescence-based activity assay. The assay measures the conversion of L-arginine into L-ornithine by a decrease in fluorescent signal due to quenching of a fluorescent probe, Arginase Gold. This way, inhibition of Arginase-1 results in a gain of signal when compared with the uninhibited enzyme. Side-by-side profiling of reference inhibitors in the fluorescence-based assay and a colorimetric urea formation assay revealed similar potencies and the same potency rank order among the two assay formats. The fluorescence-based assay was successfully automated for high-throughput screening of a small-molecule library in 384-well format with a good Z’-factor and hit confirmation rate. Finally, we show that the assay can be used to study the binding kinetics of inhibitors.

Keywords
Arginase, L-arginine, high-throughput screening, cancer immunotherapy

Introduction
Although immunotherapeutics targeting the immune checkpoints CTLA-4, PD-1, and PD-L1 have contributed substantially to recent progress in cancer treatment, a considerable proportion of patients remains unresponsive.¹ The enzyme Arginase-1, converting the amino acid L-arginine into L-ornithine and urea, is a promising new drug target for cancer immunotherapy, based on its role in the regulation of T-cell activity in the tumor microenvironment.²,³ Arginase-1 secreted by myeloid cells in the tumor microenvironment depletes local L-arginine, causing T-cell anergy and the suppression of T-cell and natural killer cell proliferation.²,³

Arginase enzyme activity is commonly determined by measuring the rate of urea formation in a colorimetric assay.₄⁵ A disadvantage of the urea formation assay is that it requires strong acidic solutions (sulfuric acid), which are incompatible with the tubing of automated dispensers commonly used for high-throughput screening (HTS). Here we describe a novel fluorescence-based screening assay for Arginase-1 and its validation using reference inhibitors and by HTS. The assay is developed to be addition only, and consists of only two reaction steps: (1) incubation of the enzyme with compound and (2) addition of the Arginase Gold probe and the substrate L-arginine, followed by development of the reaction and measurement of the fluorescence. The fluorescence of the probe is quenched upon conversion of L-arginine into L-ornithine, resulting in a decrease of the fluorescent signal. Inhibition of Arginase-1 decreases this conversion and therefore causes a gain of the fluorescent signal compared with the uninhibited enzyme.

Materials and Methods

Inhibitors and Compound Library
2-Amino-6-boronohexanoic acid (ABH)⁶ was prepared according to patent WO 2016/037298.⁷ N⁵-hydroxy-nor-L-arginine monoacetate (nor-NOHA)⁸ was purchased from Tocris Bioscience (cat. no. 6370; Bristol, UK). Compound

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3 was synthesized according to procedures described for structure 10 from patent WO 2017/075363. The compound library screened at the Pivot Park Screening Centre (Oss, The Netherlands; www.ppscreeningcentre.com) consisted of a diverse set of 87,000 leadlike compounds from a commercial supplier (Specs, Delft, The Netherlands) supplemented with 6000 compounds from another supplier (Mera Pharmaceuticals, Kailua-Kona, HI). The incorporation of desired molecular properties as well as their synthetic tractability was ensured by the Pivot Park Screening Centre.

**Protein Expression**

Full-length human Arginase-1 (L-arginine amidinohydrolase, EC 3.5.3.1) was expressed with an N-terminal hexa-histidine tag in *Escherichia coli* and purified by affinity chromatography using Ni-NTA agarose beads (Qiagen, Venlo, The Netherlands) to 95% purity, as determined by polyacrylamide gel electrophoresis. The purified protein was desalted on a PD-10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and stored at −80 °C in aliquots in buffer containing 20% glycerol. The Michaelis-Menten parameters of the protein were characterized previously.10

**Fluorescence-Based Arginase-1 Assay**

All components of the assay were diluted in assay buffer composed of either 5 mM glycine, pH 9.5, and 0.05% Tween-20 or 10 mM NaH$_2$PO$_4$, pH 7.4, and 0.01% Tween-20. Compounds were dissolved and diluted in DMSO or in MilliQ water and then further diluted in assay buffer to the desired concentrations. In a black 384-well plate (cat. no. 3573; Corning, Corning, NY), 10 µL compound solution and 10 µL of 40 nM (pH 9.5) or 80 nM (pH 7.4) Arginase-1 were combined and incubated for 90 min at room temperature. Then, 20 µL of fluorescent probe (Arginase Gold; prepared in-house and commercially available at The Netherlands Translational Research Center B.V.; www.ntrc.nl) combined with 5 mM L-arginine (cat. no. 105000250; Acros Organics, Geel, Belgium) was added to each well, followed by incubation for 30 min (pH 9.5) or 60 min (pH 7.4) at room temperature. To determine the conversion of L-arginine into L-ornithine, the fluorescence signal was read on an EnVision 2104 Multilabel Plate Reader (PerkinElmer, Waltham, MA; excitation filter X320 and emission filter M510W). The final concentration of Arginase-1 in the assay at pH 9.5 was 10 nM; at pH 7.4, it was 20 nM. The final concentration of L-arginine was 2.5 mM at both pH conditions. All experiments were performed with duplicate measurements. The $Z'$-factor of the assay was calculated using Eq. (1), with $\sigma$ and $\mu$ as the standard deviation and mean, respectively, of the positive control samples (including all reaction components except enzyme; denoted with +) and negative control samples (including all reaction components; denoted with −).

$$Z'\text{-factor} = 1 - \frac{3(\sigma_+ + \sigma_-)}{|\mu_+ - \mu_-|}$$  \hfill (1)

Dose-response curves were fit in XLFit (IDBS, Guildford, UK) with a four-parameter logistic regression to determine the IC$_{50}$ values. IC$_{50}$ values were then converted to inhibition constants ($K_i$) using the Cheng-Prusoff equation (Eq. [2]) with [S] as the L-arginine concentration and $K_M$ as the Michaelis constant of Arginase-1 for L-arginine.

$$K_i = \frac{IC_{50}}{1 + ([S] / K_M)}$$  \hfill (2)

The average $Z'$-factor of the assay at pH 9.5 was 0.70 ± 0.05, and the average signal-to-background ratio (S/B) was 1.67 ± 0.11 when performed on the lab bench. The assay was sensitive to the addition of 0.1% DMSO with a slight drop in assay performance (average $Z'$-factor of 0.67 ± 0.05 vs. 0.73 ± 0.04 with and without 0.1% DMSO, respectively), whereas the S/B remained identical for both conditions. At pH 7.4, the average $Z'$-factor was 0.62 ± 0.08, with an average S/B of 1.52 ± 0.11.

**Colorimetric Urea Formation Assay**

The colorimetric urea formation assay was performed as previously described, with the exception of the assay buffers, which were composed of either 5 mM glycine, pH 9.5, and 0.05% Tween-20 or 5 mM NaH$_2$PO$_4$, pH 7.4, and 0.05% Tween-20.

**High-Throughput Screening**

The diversity compound library was screened using the Arginase-1 activity assay at pH 9.5 on a HighRes Biosolutions robotic system (Manchester, UK). Compounds were dissolved at 2 mM in DMSO, and 40 nL of the compound solutions was transferred to the assay plates using an Echo Liquid Handler (Labcyte, San Jose, CA). The compounds were diluted by addition of 10 µL assay buffer (5 mM glycine, pH 9.5, and 0.05% Tween-20) using a Flexdrop dispenser (PerkinElmer). The assay was further executed as described above, with all solutions added using the Flexdrop dispenser. Confirmation of active compounds was performed by plating the compounds using Echo acoustic dispensers and performing the assay manually on the bench. To determine potential interference by autofluorescent compounds, the background fluorescence signal was read prior to addition of the fluorescent probe. Alternatively, to correct
for any interference of the library compounds in the assay, a
deselection assay was performed with assay buffer added to
all wells of the plates instead of the enzyme solution.

Assay quality was monitored by determining the \( Z' \)-
factor and S/B for each 384-well plate. The Z-score for
Arginase-1 inhibition in each well was calculated using Eq.
(3) with \( x \) as the sample value and with \( \mu \) and \( \sigma \) as
the mean and standard deviation, respectively, of the negative
control samples (including all reaction components).

\[
Z\text{-score} = \frac{x - \mu}{\sigma}
\] (3)

The robustness of the assay was monitored by measuring
dose-response curves of the inhibitor ABH throughout the
screening campaign. Autofluorescent compounds and com-
ounds interacting with the fluorescent probe were dese-
lected based on the criteria that the increase in either the
fluorescent background signal or the fluorescent signal in
the absence of enzyme relative to the control samples must
be less than 25% of the assay window (i.e., the difference
between the minimal and maximal fluorescent signals in the
complete assay).

**Results**

**Fluorescence-Based Arginase-1 Activity Assay**

Initial development of the fluorescence-based activity assay
was performed at pH 9.5, which is the pH optimum of
human Arginase-1. The assay was subsequently optimized
at both pH 9.5 and physiological pH of 7.4 with regard to
the enzyme concentration and reaction time. The L-arginine
concentration was chosen at 2.5 mM for both pH values,
which roughly equals \( K_M \) at pH 7.4 and half of \( K_M \) at pH
9.5.\(^{10}\) The fluorescent response of the assay was confirmed
to be linear up to this concentration at both pH values by
measurement of a calibration curve consisting of decreasing
L-arginine concentrations combined with increasing
L-ornithine concentrations (Fig. 1A, B). The enzyme con-
centration and reaction time were optimized for the assay to
be within the linear range of substrate conversion while still
maintaining a sufficient assay window (Fig. 1C, D). The
Arginase-1 concentration and reaction time were chosen at
10 nM and 30 min at pH 9.5 and at 20 nM and 60 min at pH
7.4, respectively. The higher enzyme concentration and lon-
ger reaction time required at pH 7.4 compared with pH 9.5
can be attributed to the lower catalytic activity of Arginase-1
at the lower pH (Fig. 1E, F). Finally, the preincubation time
was set at 90 min to allow for slow-binding inhibitors to
reach binding equilibrium. Nonetheless, both the preincu-
bation time and reaction time can be varied. By variation
of the reaction time, the assay can also be performed as a
kinetic assay instead of an endpoint assay.

**Pharmacology and HTS**

The pharmacology of the assay was validated by testing
three reference inhibitors and comparing their potencies
with those determined in a colorimetric urea detection
assay. The chemical structures of the three compounds are
shown in Figure 2. ABH\(^6\) and nor-NOHA\(^8\) are two different
synthetic chemical derivatives of L-arginine and are wildly
used reference inhibitors of Arginase-1. Compound 3 is a
mixture of the Arginase-1 inhibitor CB-1158 (INCB001158),
which is currently in clinical trials,\(^3\) and one of its diaster-
omers. Figure 3 shows representative dose-response curves
of the three compounds in the assay performed at pH 9.5
and at pH 7.4. The inhibition constants (\( K_i \)) are compared
with those determined in the colorimetric urea assay (Table
1). All \( K_i \) values showed maximally a 2.5-fold difference
between the two assay formats (Table 1). Moreover, the
inhibitors maintained the same potency rank order in both
assays. These results confirm the correct assay pharmacol-
ogy of the fluorescence-based assay.

To investigate the applicability of the assay in HTS, a
93,000-compound diversity library was screened for inhibi-
tors of Arginase-1 on a fully automated robotic system at
the Pivot Park Screening Centre. The compounds were
screened in 384-well assay plates at a concentration of 2 \( \mu \)M
with a final DMSO concentration of 0.1%. The average \( Z' \)-
factor of the screen was 0.59 with an S/B of 1.64 (Fig. 4). A
consistent \( K_i \) of 87 nM (95% confidence interval: 64–119
nM) was measured for the reference inhibitor ABH through-
out the screening campaign, which was highly similar to the
\( K_i \) value of 72 nM measured on the bench (Table 1).
Although this demonstrates that the screen was sensitive,
we were able to identify only 62 compounds with an effect
\( \geq 50\% \) at 2 \( \mu \)M. We therefore selected compounds showing
a signal at least three standard deviations above the negative
control mean of the respective plate (i.e., Z-score \( \geq 3 \)),
which corresponds to a roughly 20% to 25% effect, result-
ing in the identification of 621 hits (0.7% hit rate). These
compounds were retested at a single test concentration of 2
\( \mu \)M in an assay with an average \( Z' \)-factor of 0.76 and S/B of
1.8. Of the 621 hits, 48% could be confirmed. The 297 con-
firmation hits were supplemented with 49 compounds struc-
turally related to the hits (by in-silico criteria) from the same
library. The resulting 346 compounds were tested in dose-
response curves in the fluorescence-based assay. To exam-
ine the compounds for interfering properties, such as
autofluorescence or the ability to interact with the fluores-
cent probe, the same assay was also performed in the
absence of enzyme. Furthermore, all 346 compounds were
analyzed in the colorimetric urea assay as an orthogonal
assay. Of the 346 compounds, 224 compounds were dese-
lected based on displaying either autofluorescence or inter-
action with the fluorescent probe, or both. Based on the
analysis of all obtained dose-response curves and medicinal
Figure 1. Development and optimization of the fluorescence-based Arginase-1 activity assay. (A) Combined L-arginine and L-ornithine calibration curve at pH 9.5 and (B) pH 7.4 demonstrating that the fluorescent signal is linearly proportional to the L-arginine and L-ornithine concentrations within the measuring range of the assay. The concentrations present in the calibration curve correspond to the concentrations that can occur in the assay when performed with a starting concentration of 2.5 mM L-arginine, which is converted by Arginase-1 into L-ornithine. (C) Time-course of Arginase-1 activity at different enzyme concentrations after addition of 2.5 mM L-arginine and the fluorescent probe at pH 9.5 and (D) at pH 7.4 measured in real time. The fluorescence signal in panels A and B is subtracted with the background signal measured in the absence of Arginase-1. (E) Correlation between the reaction velocity and enzyme concentration at pH 9.5 and (F) at pH 7.4. The reaction velocity of 80 nM Arginase-1 at pH 9.5 was too fast to be determined accurately.

Figure 2. Chemical structures of the Arginase-1 inhibitors used in this study. The structure shown for compound 3 is that of the active diastereomer CB-1158.3,10
Table 1. Inhibition Constants of Inhibitors in the Arginase-1 Fluorescence-Based and Colorimetric Urea Formation Assays at pH 9.5 and pH 7.4.a

| Inhibitor   | pH 9.5 Assay | pH 7.4 Assay |
|-------------|--------------|--------------|
|             | Arginase-1   | Urea         | Arginase-1    | Urea         |
|             | $K_i$ (nM) (95% CI) | $K_i$ (nM) (95% CI) | $K_i$ (nM) (95% CI) | $K_i$ (nM) (95% CI) |
| ABH         | 72 (48–107)  | 30 (24–38)   | 265 (216–327) | 156 (132–185) |
| nor-NOHA    | 123 (108–141)| 80 (70–92)   | 57 (48–67)    | 50 (46–54)    |
| Compound 3  | 55 (41–72)   | 31 (23–42)   | 9.5 (8.5–10.5)| 4.9 (4.2–5.8) |

The corresponding IC$_{50}$ values are listed in Supplementary Table S1. All values were determined in either three or four independent experiments. $K_i$ values of individual experiments were averaged using their respective $pK_i$ values. ABH, 2-amino-6-boronohexanoic acid; CI, confidence interval; nor-NOHA, $N_w$-hydroxy-nor-L-arginine monoacetate.

**Chemistry expertise**, 13 compounds were ordered from a commercial vendor and retested at concentrations up to 1 and 10 mM, respectively, in the fluorescence-based and colorimetric urea formation assays. Because only minimal activity could be measured for these hits, they were not further pursued for medicinal chemistry optimization. Despite this result, we conclude that, based on the favorable $Z'$-factor and the consistent $K_i$ value of ABH in the screen, the Arginase Gold assay is robust and suitable for HTS.

**Real-Time Kinetics**

The fluorescence-based assay format has no final step in which the signal needs to be developed. Instead, the fluorescent signal can be continuously monitored. However, in some cases, it might be desirable to stop the assay after a set amount of time. For this, we studied various reaction stop protocols, such as acidic pH and reducing conditions. It appeared that addition of tris(2-carboxyethyl)phosphine (TCEP) to a final concentration of 1 mM was suitable to stop all Arginase-1 activity without sacrificing the $Z'$-factor of the assay. The continuous readout also generates the possibility of following the reaction in real time, thereby allowing the kinetics of inhibitor binding to be studied. The assay was therefore also developed to allow monitoring of the reaction progress of L-arginine conversion by Arginase-1 in real time. To determine whether the reaction time in the presence of L-arginine has an effect on the measured $K_i$ values, the inhibition of Arginase-1 was monitored as a function of the reaction time (Fig. 5). The potencies of nor-NOHA and compound 3 decreased only slightly over time at pH 9.5 and pH 7.4 (Fig. 5). The same was true for ABH at pH 7.4 (Fig. 5). In contrast, for ABH at pH 9.5, a more considerable decrease in potency as a function of reaction time was found, which may be explained by a decreased stability of the compound at increased pH. Nonetheless, considering the stable profile of nor-NOHA and compound 3 at both pH values as well as ABH at pH 7.4, these results indicate that the assay reagents do not deteriorate during the times used for the assay and that therefore the assay is suitable for performing kinetic inhibition experiments.

**Discussion**

Despite the renewed interest in Arginase-1 as a therapeutic drug target, the number of different chemotypes described in scientific publications and patents remains rather low.
Early inhibitors are analogues of the substrate L-arginine, such as the boronic acid derivative ABH. Macromolecular co-crystal structures of Arginase-1 and inhibitors have enabled the development of more potent inhibitors, such as CB-1158, which is currently investigated in phase I/II clinical trials. The primary focus on rational design and structure-based medicinal chemistry approaches may in part be related to the lack of suitable assays for HTS. Previously reported assays are generally poorly compatible with robotic screening because of the requirement of multiple reaction steps, harsh assay conditions, the use of radioactive substrate, or the use of low-turnover substrates other than L-arginine.

For Arginase-2, an automated mass spectrometry method has been described, referred to as RapidFire mass spectrometry (RapidFire-MS), which was used to screen a library of several thousands of small-molecule fragments. Mass spectrometry is a label-free method, and therefore,
technology interferences are rare. However, disadvantages of RapidFire-MS in comparison with probe-based screening methods include the requirement of specifically trained operators, the high price of equipment, and the cost of integration. Furthermore, the assay throughput is much lower. Cycle times reported for RapidFire-MS start at 5 to 10 s per sample, which equals about 1 h per 384-well plate, whereas the RapidFire Arginase-2 screen was performed with a cycle time of >18 s per sample. In contrast, the Envision multimode reader used in our studies measures 384 samples in one 384-well plate within 1 min.

The fluorescence-based activity assay for Arginase-1 is shown to be robust and suitable for HTS, as evidenced by the consistent $K_i$ value of ABH measured throughout the screening campaign and the favorable $Z'$-factor. This assay will therefore be of value for the identification of novel Arginase-1 inhibitor scaffolds. Because enzyme inhibition results in a gain of fluorescence signal in the assay, the chance of false-positive signals by fluorescent dye interference is expected to be low. Furthermore, the assay allows deselection of autofluorescent compounds and compounds interacting with the fluorescent probe by performance of the assay in the absence of enzyme. Moreover, the availability of a stop solution (i.e., TCEP) provides additional flexibility when performing an assay on the bench or applying it for HTS. However, the effect of the stop solution on

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**Figure 5.** Inhibition constants and dose-response curves in the Arginase-1 fluorescence-based activity assay with increasing reaction times at pH 9.5 and 7.4. (A) Effect of increasing reaction time on inhibition constants ($K_i$) at pH 9.5. The reaction time indicates the incubation time after addition of L-arginine to Arginase-1 and the inhibitor, during which L-arginine can be enzymatically hydrolyzed prior to readout of the assay. For each inhibitor, the $K_i$ values were scaled to the $K_i$ value of the first time point. The data points represent the mean of two independent experiments. (B) Effect of increasing reaction time on $K_i$ values at pH 7.4. The inserts in panels A and B show the linear response of the assay in time. (C–H) Representative dose-response curves with increasing reaction time at pH 9.5 and 7.4. The data points represent the mean of two technical replicates.
the interference of compounds with the assay readout still needs to be determined. Finally, an important aspect of our assay is that it can be used to monitor the binding kinetics of inhibitors in real time.

Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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