Three assays in one well: antimalarial compound library screening

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- Metalloaminopeptidases M1, M17 and M18 are attractive new targets for malaria.
- Assay developed to identify inhibitors for M1, M17 and M18 in just one well by measuring absorbance and fluorescence over time

Introduction

Malaria is a tropical disease that is caused by infection of the protozoan parasites of the genus Plasmodium. About 1,000,000 people deaths per year are related to a malaria infection. This explains the very high research interest in finding antimalarial compounds. Three metalloaminopeptidases are associated with Malaria infection: M1, M17 and M18. All three of them are zinc exopeptidases that catalyze the cleavage of a single amino acid from the N-terminus of a protein. This activity is used by the parasites to digest hemoglobin of the host organism and is absolutely necessary for parasites survival. Compounds that inhibit the peptidases activities can be used to develop a chemotherapeutic strategy.

The nonprofit foundation Medicines for Malaria Venture (MMV) have provided a compound library (MMV400) for the research community. These compounds are known for their antimalarial activity but not necessarily the molecular targets. In this application note we introduce an assay system to identify if any of the MMV400 compounds target one or more of the malarial metallopeptidases M1, M17 and M18.

Assay Principle

To avoid wasting limited amount of compounds, a high-throughput initial screen was developed that is robust enough to narrow down active compounds. As the three enzymes use different substrates it was possible to put the three peptidases, their substrates and one of the 400 compounds into one well.

Fluorescence detection

M1/M17: cleavage of a methylcoumarin substrate results in a fluorescence signal that can be measured at 355 nm excitation and 460 nm emission.

Absorbance detection

M18: cleavage of a nitroanilide substrate results in an absorbance signal at 405 nm.

With the microplate reader from BMG LABTECH, it is possible to accurately monitor absorbance and fluorescence changes over time. All data can be evaluated after the measurements are finished using the MARS data analysis software. One-click assay calculation templates simplify data processing enormously and can be provided custom-tailored by BMG LABTECH.

Materials & Methods

- Multimode reader from BMG LABTECH
- 96-well clear plates from NUNC
- MMV400 antimalarial compound library
- L-Leucine-7-amido-4-methylcoumarin-HCl (Sigma)
- L-Glutamic acid p-nitroanilide (Sigma)

In pretests it was shown that it is possible to use a single buffer for all three assays and that the 3 proteases do not interfere with each other’s activity. The correct concentrations and volumes for the proteases and the substrates were optimized to have a final assay volume of 200 μl including 20 μl of 1 mM compound in 100 % DMSO.

The MMV400 library consists of five 96-well plates containing 400 compounds with nothing in column 1 and 12. The 16 empty wells were used as controls. In 12 control wells all three proteases (PotP) and substrates (PotS) were pipetted but no compounds. In three control wells only the single proteases, M1 or M17 or M18 and the substrate mix (PotS) were present and the remaining control well contained PotP, PotS and a pot of inhibitors (PotI) that were created in our laboratory. The PotP and PotS stock solution were prepared freshly.

Pipetting order

- Add DMSO and PotI to appropriate wells
- Add 80 μl of buffer to all wells with a multichannel pipette
- Leave for 5 min at room temperature
- Add individual proteins (50 μl) to 3 control wells, add PotP (50 μl) to all remaining wells
- Incubate 10 minutes at 37°C with lid on the plate.
- Add PotS (50 μl) to all wells with a multichannel pipette
- Read assay on the microplate reader, pre-warmed at 37°C

Instruments settings

<table>
<thead>
<tr>
<th>Detection mode</th>
<th>Fluorescence and Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reading mode</td>
<td>Plate mode kinetic</td>
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<td>Number of cycles</td>
<td>25</td>
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<tr>
<td>Cycle time</td>
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</tbody>
</table>

Optic settings

M1/M17 detection: Ex355/Em460
M18 detection: 405-10

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Results & Discussion

Fig. 1 shows the change in fluorescence over time related to M1/M17 activity.

Accuracy of the screen is dependent on the PotP+PotS activity alone (without compounds). As expected these controls gave the highest increase of signal over time. The negative control containing inhibitors shows no significant increase of fluorescence over time. Active compounds can be identified by comparing the linear increase/min to the both controls. The lower the fluorescence increase over time the more active is the compound. 25 compounds were identified as having activity against M1 or M17. Some of them are shown in figure 2.

Fig. 2: Screenings results for the fluorescent M1/M17 inhibitor screening. All compound (Cpd) data is compared to the positive control (PC) and the negative control (NC).

A secondary screen was performed to confirm results and identify M1 or M17 as the target. Final results show that only 2 compounds were not inhibitory in the secondary screen. That leads to an 80% success rate for the Pot Assay.

No inhibitor for M18 was identified (Fig. 3). This was not surprising as M18 is a highly specific enzyme and 400 compounds is not a big library.

Conclusion

With the help of the BMG LABTECH multimode microplate reader it was possible to develop a robust assay to determine active compounds against the metallo-aminopeptidases M1, M17 and M18. By utilizing the instrument to read absorbance and fluorescence over time three assays could be performed in just one well saving limited amount of compounds. With this approach it was possible to screen the whole MMV400 library in just one day with only minimal lab work involved.