A Robust Multiplex Assay by Mass Spectrometry for HTS: CD73 Inhibitors Identification

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Overview

- Multiplex assay is a good way to improve throughput in HTS context
- Combining of four conditions in one well allows to analyze 1536 samples in 384 well-plate
- This approach has been successfully applied in RapidFire-MS/MS to the identification of CD73 inhibitors
- It allowed to screen more than 760,000 compounds, leading to 1775 hits, including 300 compounds with IC_{50} in nanomolar range

Introduction

In high-throughput screening (HTS) of small molecules, multiplex assay could allow the combination of four different 384-well plates in a single one plate, leading to the analysis of 1536 samples in the reading time of only 384 wells. This approach was first imagined and developed in RapidFire-MS/MS (RF-MS/MS) by Sanofi's team, and then used in a collaborative work between Sanofi and Evotec, aiming to identify inhibitors of CD73 enzyme, which is involved in immunosuppressive mechanisms, impeding antitumor immunity.

CD73 catalyzes conversion of adenosine monophosphate (AMP) to free phosphate and adenosine (ADO), which will accumulate in the extracellular microenvironment, allowing tumors to escape from immunosurveillance (Fig. 1). This CD73 driven-reaction is a rate-limiting step in the ADO pathway; therefore, CD73 is considered as a potential drug target. Although several pharmaceutical companies and academic groups are actively exploring targeting this enzyme using antibody-based antagonists, using small molecules to inhibit its catalytic activity is gaining interest from the drug discovery community because of their potential for better tissue penetration, better oral bioavailability, easier control of treatment dosage, and lower cost of development.

In this way, biochemical assays are needed to study the CD73 enzymatic activity, and to characterize potential inhibitors. This need lead Sanofi's collaborators to imagine the multiplex approach presented here, on a mass spectrometry-based assay. Development and validation of the strategy, but also application on CD73 HTS campaign are detailed below.

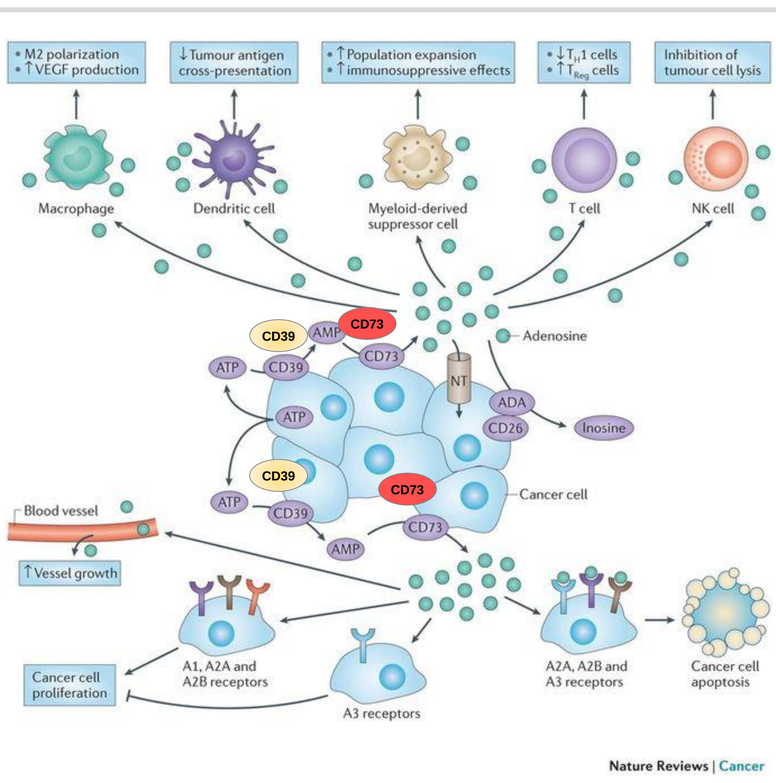

Figure 1: The role of CD73 in the immunosuppressive effect of ADO from tumor cells. Tumor cells will secrete adenosine triphosphate (ATP), that is then transformed in Adenosine monophosphate (AMP) by tumor-expressed CD39. AMP is finally converted in Adenosine (ADO) by CD73 enzyme also expressed by tumor cells, and this ADO will interact with all of the immunity cells to mask the presence of the cancer cells. This pathway will promote the tumor cell proliferation, and the CD73 driven-reaction is a rate-limiting step in this ADO pathway; therefore, CD73 enzyme is considered a potential drug target.

Methods

- **RF-MS/MS assay using recombinant CD73:** Using $[^{15}N]_5$-AMP as a substrate, assay buffer optimization was performed by Sanofi's team, to finally get a reaction buffer with 50mM Tris-HCl pH 7.4, 0.005% TX-100, and 1% DMSO. Time course reaction was performed with different CD73 soluble enzyme concentrations (from 0.03 to 0.1 nM). CD73 reaction was monitored by quantifying $[^{15}N]_5$-ADO converted from $[^{15}N]_5$-AMP. Optimized reactions were conducted in a 20µL in a 384-wells plate. V_{max} and K_M were determined with 0.075nM of CD73. To evaluate inhibitory activity of compounds, testing compounds were incubated with CD73 for 1h at room temperature before reaction was initiated by adding substrate at 5µM final concentration. CD73 reactions were quenched after 8-10 min with 20µL of 1.6µM ADO and 2% formic acid in water. Reaction mixture was then diluted 20-fold with water and subjected to RF-MS/MS analysis.
- **High-throughput screening for CD73 inhibitors:** A collection of 762,400 compounds was screened using the multiplex approach illustrated in Figure 1. An automatized flow integrating a Multidrop Combi (Thermo Scientific), an Agilent Bravo and a Beckman platform was established to process assay plates in an HTS mode. CD73 reactions (40.3 µL) with different isotope-labeled AMPs ($[^{15}N]_5$-AMP, $[^{13}C]_{10}[^{15}N]_5$-AMP, $[^{2}H]_2$-AMP, or $[^{13}C]_5$-AMP) were carried out in separate 384-well assay plates (Greiner Bio-One, cat. 781280). Each reaction solution is composed of 0.075nM recombinant CD73 and 10µM of isotope-labeled AMP in 50 mM Tris-HCl pH 7.4, 0.005% TX-100 and 1% DMSO. Each 384-well plate included 16 positive controls (reactions without compound) and 32 negative controls (pre-quenched reactions). The mean of these controls was used to calculate the percent inhibition (%I) of compounds. The primary screen was conducted at 10 µM compound.

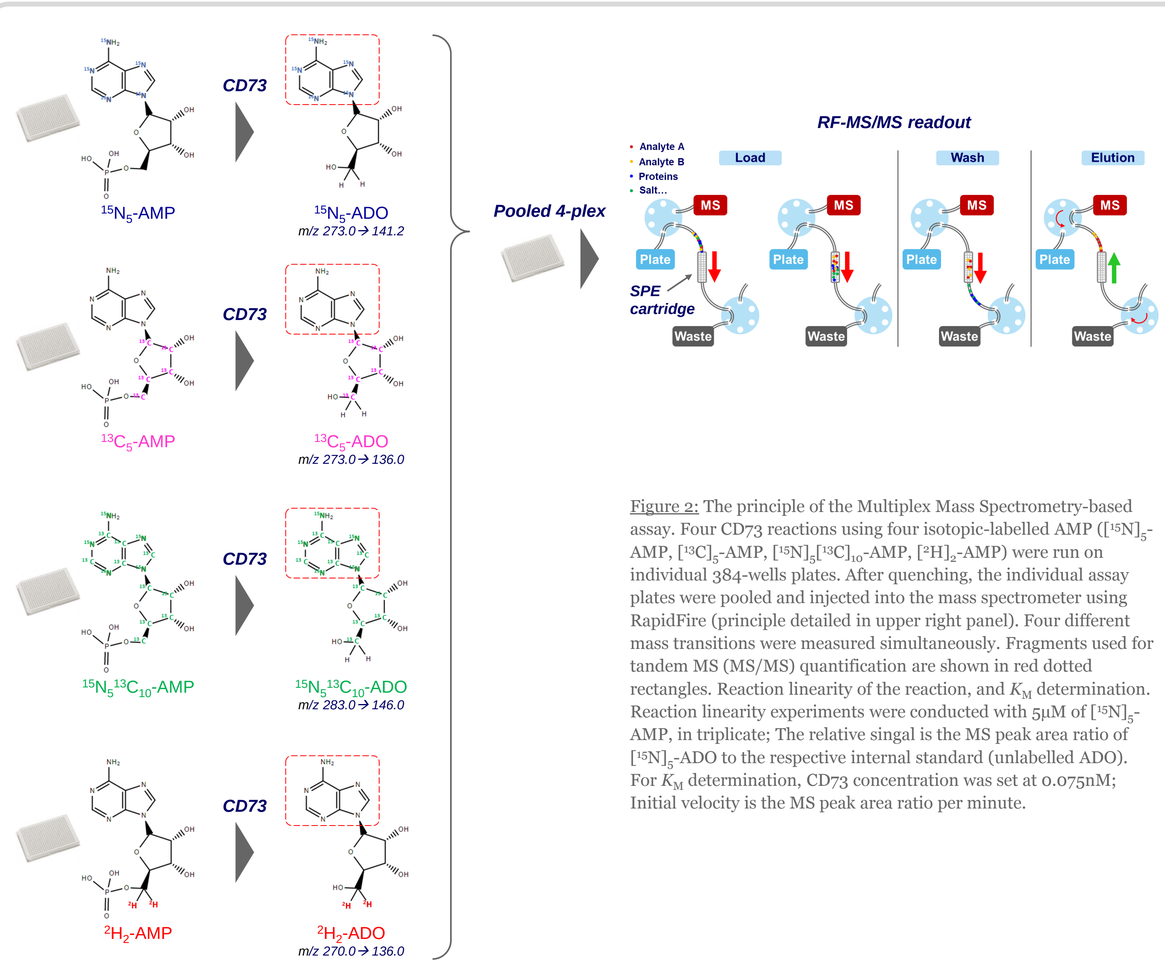

Figure 2: The principle of the Multiplex Mass Spectrometry-based assay. Four CD73 reactions using four isotopic-labelled AMP ($[^{15}N]_5$-AMP, $[^{13}C]_5$-AMP, $[^{15}N]_5[^{13}C]_{10}$-AMP, $[^{2}H]_2$-AMP) were run on individual 384-wells plates. After quenching, the individual assay plates were pooled and injected into the mass spectrometer using RapidFire (principle detailed in upper right panel). Four different mass transitions were measured simultaneously. Fragments used for tandem MS (MS/MS) quantification are shown in red dotted rectangles. Reaction linearity of the reaction, and K_M determination. Reaction linearity experiments were conducted with 5µM of $[^{15}N]_5$-AMP, in triplicate; The relative singal is the MS peak area ratio of $[^{15}N]_5$-ADO to the respective internal standard (unlabelled ADO). For K_M determination, CD73 concentration was set at 0.075nM; Initial velocity is the MS peak area ratio per minute.

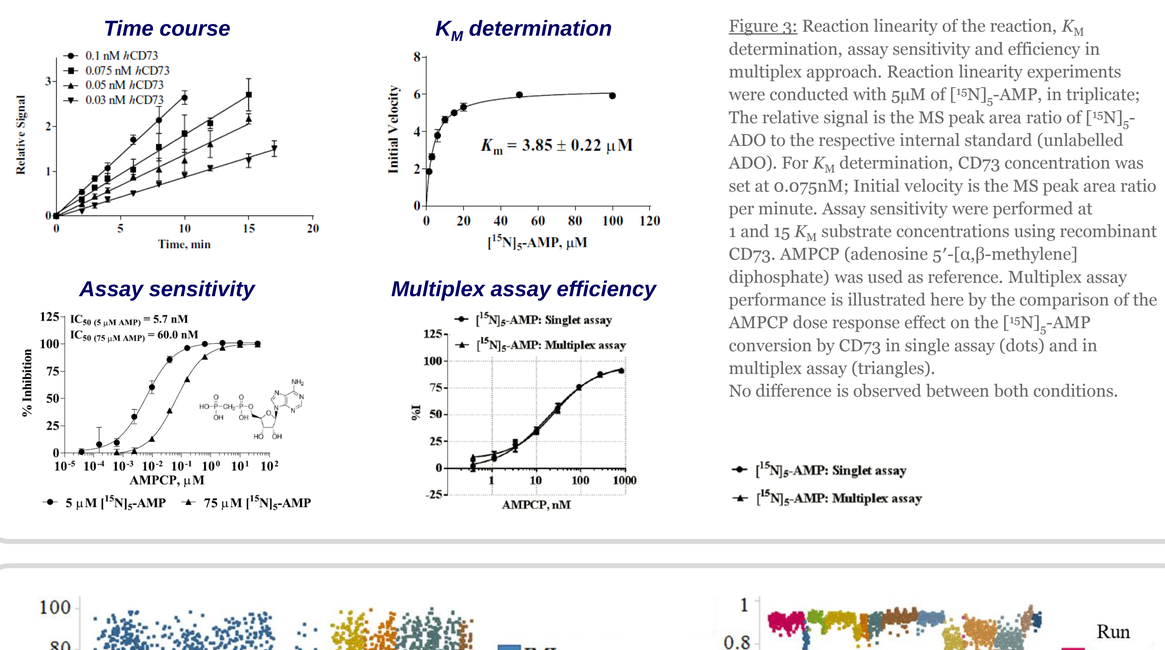

Figure 3: Reaction linearity of the reaction, K_M determination, assay sensitivity and efficiency in multiplex approach. Reaction linearity experiments were conducted with 5µM of $[^{15}N]_5$-AMP, in triplicate; The relative signal is the MS peak area ratio of $[^{15}N]_5$-ADO to the respective internal standard (unlabelled ADO). For K_M determination, CD73 concentration was set at 0.075nM; Initial velocity is the MS peak area ratio per minute. Assay sensitivity were performed at 1 and 15 K_M substrate concentrations using recombinant CD73. AMPCP (adenosine 5′-[α,β-methylene] diphosphate) was used as reference. Multiplex assay performance is illustrated here by the comparison of the AMPCP dose response effect on the $[^{15}N]_5$-AMP conversion by CD73 in single assay (dots) and in multiplex assay (triangles).
No difference is observed between both conditions.

Results

- **CD73 Assay development:** Using several isotopically labeled AMPs and an ultrafast RF-MS system, CD73 enzymatic reaction can be quantified at a speed of 38 reactions/min. Excellent injection reproducibility (STDV ~4%), signal-to-noise ratio (S/N = 700), and low sample carryover (<5%) were achieved. After optimization of the assay buffer by Sanofi's team, only 0.075nM of CD73 enzyme was needed, reaction was linear for up to ~15 min, and the K_M of AMP was determined to be 3.85 ± 0.22 µM (Fig. 2).
  The effectiveness of reaction quenching using 1% formic acid (final 0.05% after dilution with 20 volumes of water) was confirmed by monitoring the amount of $[^{15}N]_5$-ADO for up to 12 h post quenching/dilution in water. The level of $[^{15}N]_5$-ADO remained constant for up to 12 h, indicating CD73 reaction was completely stopped following acid quenching. The stability of the reaction product ADO in post-quenching and dilution solution was evaluated by measuring $[^{15}N]_5$-ADO throughout time. When assay plates were kept on the RF system for 24 h at RT, no loss of ADO was observed. The performance of the multiplex assay format using $[^{15}N]_5$-AMP, $[^{13}C]_{10}[^{15}N]_5$-AMP, $[^{2}H]_2$-AMP, and $[^{13}C]_5$-AMP was evaluated by determining the IC_{50} of AMPCP. Results showed excellent agreement with those obtained using a single-substrate assay, which are presented in Fig. 2.
- **Assay validation and HTS:** To validate the RF-MS/MS assay for HTS, a validation collection of 8000 diverse compounds was tested at 10 µM in duplicate. The resulting Z'-factor of 0.88 and correlation coefficient (R^2) of 0.66 indicated that HTS can be conducted robustly with high quality.
  HTS was conducted at 10 µM against different compound libraries (**762,400** compounds). 566,400 compounds were screened at the Evotec screening laboratory, and 196,000 compounds were screened in a Sanofi laboratory. The average hit rate was 1.02% with a %I cutoff at mean + 3 SD. As an example, the results for screening 566,400 compounds are illustrated in Fig. 4. The confirmed hits were further analyzed in 8- or 10-point compound dilution series with a dilution factor of semi-logarithmic at a top concentration of 30 µM. Among all 762,400 compounds screened, **1775** hits showed **IC_{50} < 30µM**, and **300** hits have **IC_{50} < 1µM**.

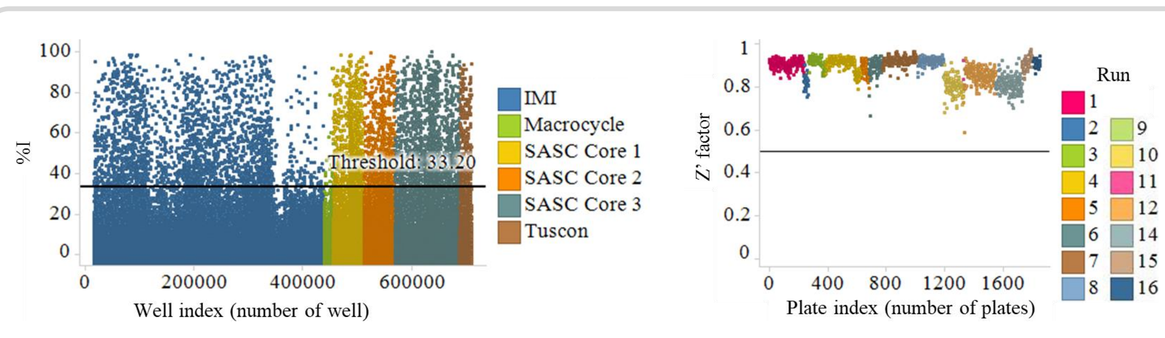

Figure 4: Primary screen results from CD73 HTS campaign performed at Evotec (with 566,400 compounds). This analysis was conducted at 10 µM of compound. A threshold (mean + 3SD) of 33.20 %I was determined, leading to an average hit rate of 1.02%.

Conclusions

In High-throughput screening, the capacity to analyze rapidly with a good accuracy is the main concern. In this way, 1536-well plates are a huge improvement compared to smaller format, such as 384-well plates, but it requires compatible readout system. For readout system only compatible with 384-well plates (or smaller), multiplex assay could be a wonderful alternative to improve screening throughput. Here, we have presented this approach imagined by Sanofi's team at Cambridge (US), and then successfully applied to the identification of CD73 enzyme inhibitors. This enzyme represents an important drug target due to its crucial role in the adenosine pathway in tumor cells. Practically, we have pooled four different reactions plates in a single one, using four different isotopic-labeled adenosine monophosphate (AMP) as CD73 substrate.
The reaction conducted to 4 different isotopic-labeled adenosine (ADO) products, which can be differentiated and quantified by RapidFire-MS/MS (RF-MS/MS). This multiplex assay has been developed and validated by Sanofi and ourselves, and finally used for an HTS campaign among 762,400 compounds, leading to the identification of 1775 hits with IC_{50} <30 µM, including 300 compounds with an IC_{50} in nanomolar range.
All together, these results confirm the capacity of this multiplex approach to enhance throughput for HTS campaign, but also the ability of our assay to catch promising compounds for CD73 inhibition.

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