A NOVEL HOMOGENEOUS TIME-RESOLVED FLUORESCENCE METHODOLOGY THAT IDENTIFIES THE FIRST FAMILY OF NATURAL PRODUCTS OF MICROBIAL ORIGIN AGAINST THE PD-1/PD-L1 IMMUNO ONCOTARGET

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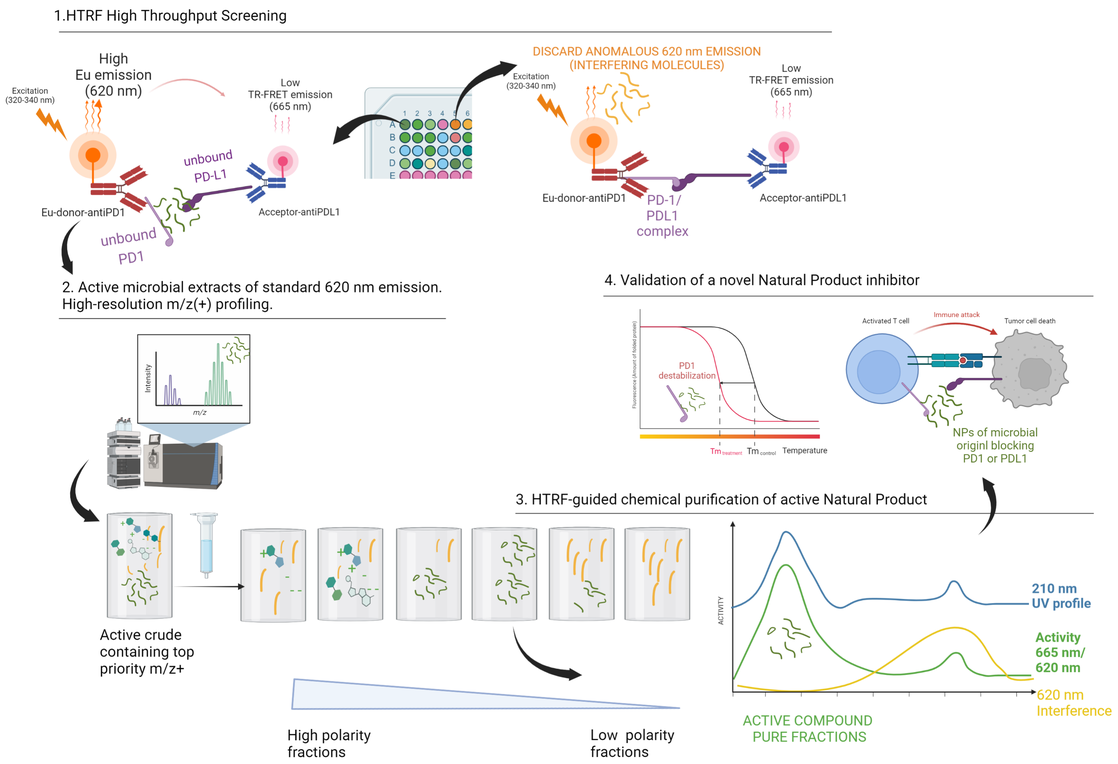
INTRODUCTION

PD-1 is a checkpoint protein on immune T cells. As such, T cell´s PD-1 binds to the PD-L1 receptor, of either cancer or normal cells, to trigger an intercellular signaling event that prevents the corresponding immune attack. Cancer cells tend to overexpress PD-L1 to enhance tumor expansion. Even though there are anti-PD-1 and anti-PD-L1 therapeutic antibodies clinically validated to date [3], these therapeutics present adverse immunological issues, implying there is a need for alternative inhibitors that could present a better immunological profile [5]. We present herein the first high throughput assay for the screening of microbial extracts that is based on the homogeneous time-resolved fluorescence methodology [1], [2]. We apply such a novel methodology to identify PD-1/PD-L1 inhibitors from a library of fungal and bacterial extracts of maximized diversity [4]. As result of this successful proof of concept against filtered and concentrated crudes from 117 strains, we have detected, isolated and characterized the first family of PD-1/PD-L1 inhibitors of microbial origin. These inhibitors present a distinctive molecular mechanism of action based on the destabilization of the PD-1 protein. We also show evidence that the most potent member of family of compounds has scope as new inhibitor of immune check-point PD-1/PD-L1 by demonstrating cellular activity in a cellular blockade assay [6].

METHODS



Filtered and concentrated crude extracts were screened by our homogeneous time-resolved fluorescence (HTRF) assay, whose activity is represented as a 665 nm emission/620 emission ratio and expressed as relative activity with respect to BMS-1 control. Subsequent normalization of the 620 nm emission from the donor, which should remain invariable with respect to BMS-1 control, allows the deconvolution of the interfering crudes from the active ones. The quick labelling of the active crudes by high resolution mass-spectrometry accelerates the identification of known m/z(+) responsible for the activity by matching them with known molecular formulas available in our databases. Further HTRF-guided chemical purification at the preparative scale leads to the isolation of the active Natural Product for further characterization against the PD-1/PD-L1 immune oncotarget comprising HTRF, AlphaScreen, Thermal shift assay, cellular toxicity assay and PD-1/PD-L1 cellular blockade assay [1],[2],[6].

CONCLUSIONS

- Screening of microbial extracts becomes efficient by our novel homogeneous time-resolved fluorescence approach.
- This novel screening application overcomes the limitations of pioneering fluorescence polarization efforts.
- The first family of bacterial natural products active against PD-1/PD-L1 inhibitors is reported.
- TSA and alphaLISA assays confirms the destabilization of PD1-target as MoA for the disruption of PD1/PD-L2 complex for the compounds-01-03.
- Compound-01 yielded a relative activity of 23.0 ± 1.5 at 6 µM with respect to vehicle (1% DMSO) as 0% activity in the PD-1/PD-L1 cellular blockade assay.
- Comparison between the activity in the HTRF assay and the cellular blockade assay suggests a higher relative potency of compound-01 with respect to compound-02 and compound-03.

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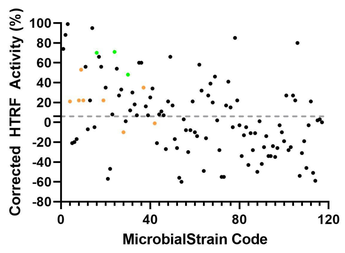
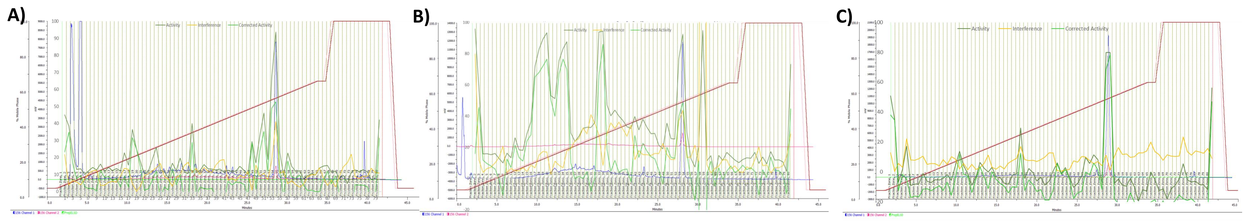
RESULTS

- **A summary of the Homogeneous Time-Resolved Fluorescence (HTRF) screening against a collection of 117 microbial extracts of maximized diversity.**



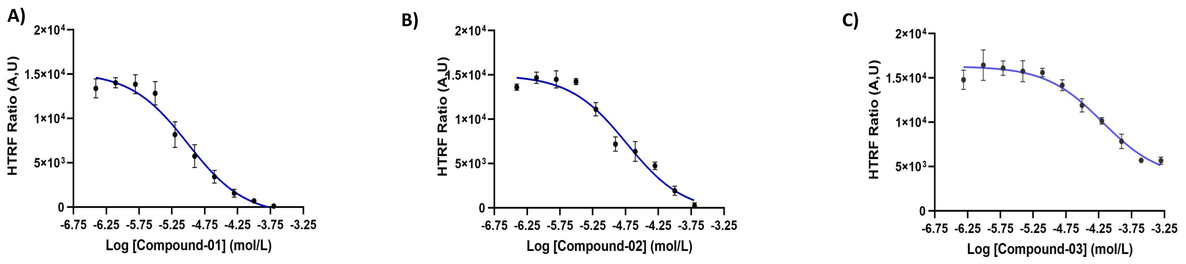
Representation of the microbial extracts in the x axis and the correction of the raw inhibitory activity (665 nm/620 nm emission ratio) by removing the contribution of sample interference in the 620 nm emission channel and defined as 0% for BMS-1 positive control and as 100% for Coomassie blue interference standard. Horizontal line in grey represents the average values for each population. The green dots represent the microbial fermentations from which the family of active Compound-01-03 have been isolated. Orange dots represent interference extracts [6].

- **HTRF-guided chemical purification of Compounds-01-03:**



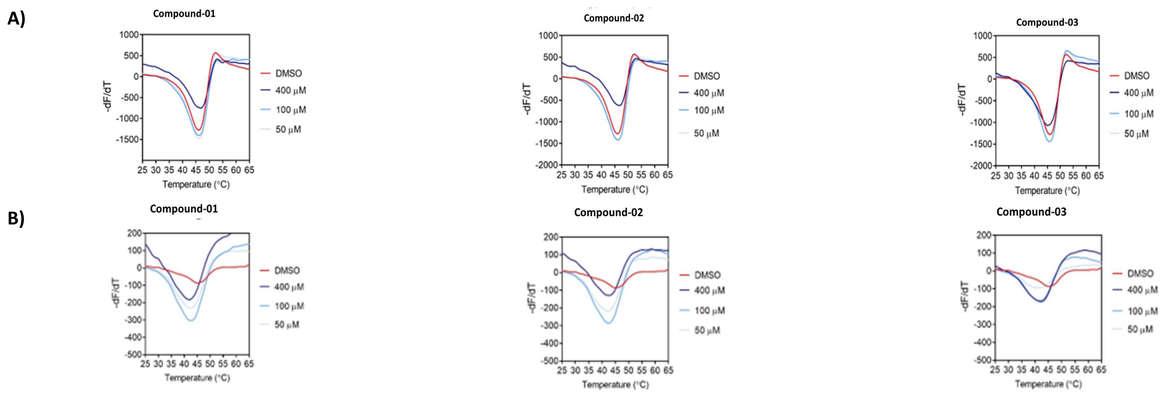
Preparative HPLC displaying the UV profile (blue line), the HTRF activity profile (dark green line), the HTRF corrected activity (green line) and the interference profile (yellow line). A) Compound-01, B) Compound-02; C) Compound-03 [6].

- **HTRF confirmation of Compounds-01-03 validates the screening setup:**



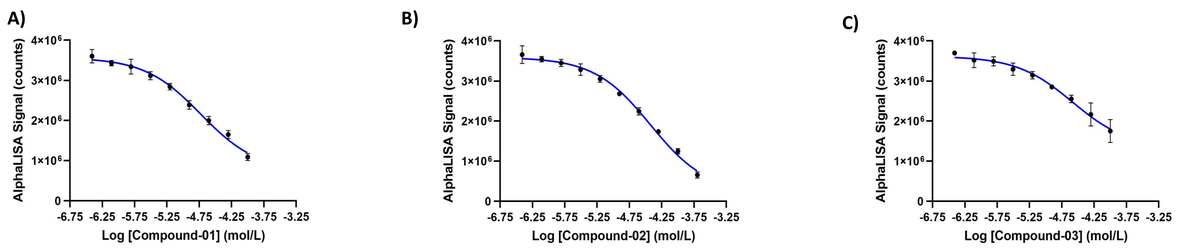
A) Compound-01 (IC_{50} = 9.8 µM ± 4.6 µM), B) Compound-02 (IC_{50} = 18.6 µM ± 13.7 µM), C) Compound-03 (IC_{50} = 64.4 µM ± 30.7 µM). All curves were obtained at 1% DMSO final, except for C, which was further optimized at 5% DMSO because of compound solubility issues [6].

- **Thermal shift experiments against PD-1 and PD-L1 confirms the MoA of Compounds-01-03**



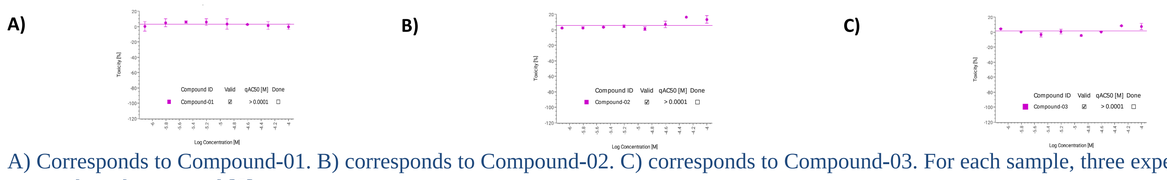
Melting curves reporting for the stabilization or destabilization ΔTm (ºC) of PD-L1 or PD-1 upon binding to Compounds-01-03 with respect to vehicle alone at an equivalent DMSO concentration of 5%. Panel A corresponds to PD-L1 thermograms above demonstrating not meaningful ΔTm (ºC) = 0.1 ºC. Panel B corresponds to PD-1 thermograms below confirming ΔTm (ºC) < -1.7 degrees [6] for Compounds-01-03 and therefore that the destabilization of PD-1 corresponds with the inhibition of the PD-1/PD-L1 complex.

- **Orthogonal confirmation of the MOA for Compounds-01-03. AlphaLISA PD-1/PD-L2 assay.**



A) Compound-01 (IC_{50} = 18.8 µM ± 8.2 µM), B) Compound-02 (IC_{50} = 37.7 µM ± 12.1 µM), C) Compound-03 (IC_{50} = 26.1 µM ± 15.8 µM). All curves were obtained at 1% DMSO final. [6]

- **Toxicity assay on Jurkat cells.**



A) Corresponds to Compound-01. B) corresponds to Compound-02. C) corresponds to Compound-03. For each sample, three experimental repeats have been tested [6].

- **Cellular blockade activity in correlation with the PD-1/PD-L1 activity in protein-based assays**

Assay	Control (Vehicle)	Positive Control	Compound-01	Compound-02	Compound-03
HTRF (IC50, µM)[1]	0	0.30± 0.05	**9.8 ± 4.6**	18.6 ± 13.7	64.4 ± 30.7
Cellular blockade[2] (relative vehicle, 0%)	0	102.0 ± 1.9	**23.0 ± 1.5**	1.0 ± 2.8	5.5 ± 1.0
alphaLISA[3] PD-1/PD-L2 (IC50, µM)	0	0.4 ± 0.2	**18.8 ± 8.2**	37.7 ± 12.1	26.1 ± 15.8
TSA[4] PD-1 (ΔTm, ºC)	0.0 º	-	**-2.1 º**	-1.7 º	-3.7 º
TSA[5] PD-L1 (ΔTm, ºC)	0.0 º	6.5 º	**-0.1 º**	0.1 º	-0.1 º

Characterization of Compound-01,-02 and -03 by HTRF, cellular blockade assay, AlphaLISA PD-1/PD-L2 and TSA. [1]Potency (IC_{50}) in the HTRF assay, BMS-1 10µM is used as positive control. [2]Relative potency in the cellular blockade assay at compound concentration of 6 µM is referred to the inhibition of Durvalumab 2.9µg/mL as 100% activity. [3]Potency in the AlphaLISA assay, Nivolumab 10µg/mL is used as control. [4]ΔTm values are shown for a compound concentration of 50 micromolar in 5% DMSO. [5]ΔTm values are shown for a compound concentration of 50 micromolar is referred to the stabilizing effect of BMS-1166 as positive control.[6].