Cell-Based Identification of New IDO1 Modulator Chemotypes

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Abstract: The immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO1) strengthens cancer immune escape, and inhibition of IDO1 by means of new chemotypes and mechanisms of action is considered a promising opportunity for IDO1 inhibitor discovery. IDO1 is a cofactor-binding, redox-sensitive protein, which calls for monitoring of IDO1 activity in its native cellular environment. We developed a new, robust fluorescence-based assay amenable to high throughput, which detects kynurenine in cells. Screening of a ca. 150000-member compound library discovered unprecedented, potent IDO1 modulators with different mechanisms of action, including direct IDO1 inhibitors, regulators of IDO1 expression, and inhibitors of heme synthesis. Three IDO1-modulator chemotypes were identified that bind to apo-IDO1 and compete with the heme cofactor. Our new cell-based technology opens up novel opportunities for medicinal chemistry programs in immuno-oncology.

Cancer cells have evolved mechanisms to evade immune cell-mediated elimination and modulation of these processes is a promising approach in anti-cancer drug discovery.[1] Indoleamine-2,3-dioxygenase 1 (IDO1) is an immunoregulatory enzyme that is induced by pro-inflammatory cytokines, such as interferon gamma (IFNγ).[2] In various cancers, IDO1 expression is associated with poor prognosis.[3] IDO1 is a heme-containing protein and catalyzes the conversion of L-tryptophan (Trp) to N-formylkynurenine (NFK), which is further degraded to kynurenine (Kyn).[4] IDO1-mediated depletion of Trp reduces effector T-cell proliferation, and accumulation of Kyn promotes regulatory T-cell differentiation, thus reducing anti-tumor immunity and supporting tumor progression.[5] Hence, inhibition of IDO1 may restore immune-mediated cancer cell elimination, and various chemotypes were identified as IDO1 inhibitors by means of target-based approaches.[6] Unfortunately, exploration of IDO1 inhibitors for the treatment of unresectable or metastatic melanoma has had limited success. The IDO1 inhibitor epacadostat in combination with pembrolizumab, an antibody that targets programmed cell death protein 1 (PD-1), did not outperform treatment with pembrolizumab alone. However, neither tumoral IDO1 expression of selected patients nor Kyn levels as a biomarker for IDO1 were evaluated.[7] Therefore, inhibition of IDO1 by means of new inhibitor chemotypes remains a promising opportunity to enhance immune cell-mediated elimination of cancer cells. In this context, exploration of alternative binding sites of IDO1, like allosteric inhibition or targeting apo-IDO1 may be of particular interest.[7,4]

Redox-sensitive proteins like IDO1, which are vulnerable to redox-cycling compounds, and interference of iron chelators with the heme cofactor may lead to promiscuous IDO1 inhibition.[9] Moreover, the temperature and reducing agents during pre-incubation of IDO1 with compounds can influence potency.[9a,10] Even more important, the validation of several IDO1 inhibitors from in vitro to in cellulo is challenging.[9b] These problems might be overcome by means of cell-based assays in which the protein remains in its native environment. Currently employed cell-based assays monitoring IDO1 activity utilize p-dimethylamino benzaldehyde (pDMAB),[11] high-performance liquid chromatography (HPLC),[12] NFK Green/C27,[13] or cucurbit[8]uril-dimethylamidopyrenium dication complex (MP-CB[8]).[14] HPLC-based techniques or pDMAB require a transfer of cell supernatant which is technologically challenging in screening assays. NFK Green displays a low dynamic detection range in cells,[13] and MP-CB[8]-mediated detection of Trp requires homogenous diffusion across cell membranes.[14] Thus, there is a high demand for new cell-based screening assays for identification of IDO1 inhibitor classes.

Here, we describe the discovery of highly potent IDO1 inhibitor chemotypes along with the finding of indirect IDO1 modulators by means of a newly developed cellular assay. The identified compound classes display diverse mechanisms of action. They include modulators of IDO1 expression in cells, inhibitors that interfere with the synthesis of the heme cofactor and compounds that inhibit IDO1 directly, for example, by an interaction with apo-IDO1.

To determine IDO1-mediated Kyn production in cells by means of a fluorescence readout, we employed the previously developed coumarin-based Kyn sensor 2H5 (Figure 1 A). In aqueous buffer, aldehyde 2 reversibly reacts with the aniline moiety of Kyn to yield adduct 3 (Figure 1 A).[15] As 2 has not
yet been employed in enzymatic or cell-based assays, we analyzed the spectral properties of \(2\) and \(3\) in cell culture medium in the presence of 10% fetal bovine serum (FBS). \(2\) absorbs light below 500 nm in cell culture medium and an additional absorbance peak between 525 nm and 560 nm was observed in the presence of Kyn for Schiff base \(3\) (Figure 1B). Using ex/em 555/600 nm, the sensor detected different Kyn levels in cell culture medium (Figure 1C, Figure S1). For further validation, we analyzed the production of Kyn in the human pancreas adenocarcinoma cell line BxPC3. Whereas IDO1 protein is not expressed in BxPC3 cells, stimulation with IFN\(\gamma\) induced IDO1 expression after 24 h, and 48 h were required for sufficient Kyn production (Figure 1D and Figure S1). For assay optimization, the concentration of the substrate Trp, the incubation time and the sensor concentration were varied (Figure S1), and the IDO1 inhibitors epacadostat (4) and BMS-986205 (5) were employed as controls (Figure 1E).\(^{[10b,11,16]}\) BxPC3 cells were stimulated with IFN\(\gamma\) for 48 h prior to detection of Kyn by means of sensor \(2\). Similar to Kyn detection reagent \(p\)-DMAB,\(^{[11]}\) \(2\) detected a dose-dependent decrease in Kyn levels in BxPC3 cells by epacadostat with a half-maximal inhibitory concentration (IC\(_{50}\)) of 198 ± 9 nM, which is in good agreement with the IC\(_{50}\) value obtained using \(p\)-DMAB (IC\(_{50}\) = 196 ± 4 nM) (Figure 1F). Automatization and miniaturization to 384-well format successfully detected the concentration-dependent decrease in Kyn levels for both IDO1 inhibitors (IC\(_{50}\) = 102 ± 0.1 nM (4) and IC\(_{50}\) = 14 ± 0.1 nM (5)) (Figure 1G) with robust assay characteristics (Z’-factor: 0.76; signal-to-background ratio (S/B): 14). For the initial screening, the assay was further miniaturized to 1536-well format (Z’-factor: 0.53 and S/B: 14.3). Thus, we developed a robust cellular Kyn assay with a high fluorescence dynamic range that is applicable for high-throughput analysis.

Screening of a compound library of 157332 chemically diverse, commercially available and in-house synthesized compounds at 7.1 μM using the Kyn assay resulted in a hit rate of 0.62% (threshold: >50% inhibition). Compounds that reduced cell viability by more than 25% and small molecules...
with pan-assay interference (PAINS)\textsuperscript{[17]} features were excluded from further analysis (see Supporting Information). Compounds that reduce Kyn production by 70% or more were subjected to dose-response measurements. Kyn levels are mainly regulated by IDO1 enzymatic activity in tumor cells.\textsuperscript{[18]} Hit compounds with IC\textsubscript{50} values $<5\text{ \textmu m}$ were analyzed for direct modulation of IDO1 activity which revealed that only 3.9% of the compounds inhibited IDO1 activity (IC\textsubscript{50} $\geq 0.33\text{ \textmu m}$), including several chemotypes that have not been linked to IDO1 inhibition before (Figure 2A, Figure S3 and S4). Hits that did not directly inhibit IDO1 were considered indirect inhibitors (Table 1), including two groups of compounds with annotated mechanism for reduction of Kyn levels. Group 1 downregulates IDO1 expression in cells, whereas group 2 inhibits the synthesis of the IDO1 cofactor heme (Table 1). For example, JAK1/2 inhibitors like ruxolitinib potently reduced Kyn levels (Table 1) by downregulating IDO1 expression (Figure S5), which is in agreement with reduced Kyn levels in cells upon targeting the IFN-\gamma-induced JAK/STAT pathway.\textsuperscript{[4b]} Furthermore, bromodomain-containing protein 4 (BRD4) is involved in the epigenetic regulation

\begin{table}[h]
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\hline
Compound & Kyn Assay IC\textsubscript{50} [\textmu m] & Cell Count IC\textsubscript{50} [\textmu m] & Annotated Activity & Mode of Action \\
\hline
Momelotinib (CYT387) & 0.29 ± 0.06 & inactive & ATP-competitive inhibitor of Janus kinases JAK1 and JAK2 & JAK kinase inhibitors \\
Tofacitinib & 0.59 ± 0.008 & inactive & Irreversible inhibitor of Janus kinases JAK1 and JAK3 & \\
Ruxolitinib & 0.08 ± 0.005 & inactive & ATP-competitive inhibitor of Janus kinases JAK1 and JAK2 & \\
Tofacitinib citrate & 0.28 ± 0.03 & inactive & Irreversible inhibitor of Janus kinases JAK1 and JAK3 & \\
CEP-33779 & 0.45 ± 0.04 & inactive & ATP-competitive inhibitor of Janus kinase JAK2 & \\
GSK124726A & 0.01 ± 0.005 & > 2 & Inhibitor of bromodomain and extra-terminal (BET) family proteins BRD2, BRD3, and BRD4. & BRD/BET inhibitors \\
(+)-JQ1 & 0.03 ± 0.004 & > 2 & Inhibitor of BRD4 (1/2) & \\
OXT015 & 0.03 ± 0.01 & > 2 & Inhibitor of BRD2, BRD3, and BRD4 & \\
I-BET151 & 0.07 ± 0.002 & inactive & Pan BET family inhibitor & \\
M21 & 0.09 ± 0.04 & 1.24 & Proteolysis-targeting chimera (PROTAC) based on JQ-1. & \\
Inhibits proteasomal degradation of BRD4. & \\
PFI-1 & 0.51 ± 0.06 & inactive & Inhibitor of BRD4 & Heme biosynthesis inhibitors \\
PFI-1 & 0.51 ± 0.06 & inactive & Selective cellular chemical probe for BRD9 & \\
I-BRD9 & > 10 & inactive & Inhibitor of the δ-aminolevulinic acid dehydratase, & \\
which produces porphobilinogen, the precursor of heme. & \\
Succinylacetone & 0.41 ± 0.22 & inactive & Inhibitor of protoporphyrin IX ferrochelatase, & \\
which is involved in heme biosynthesis. & \\
N-methyl protoporphyrin IX & 2.61 ± 1.64 & inactive & & \\
\hline
\end{tabular}
\caption{Identified compounds with known targets that reduce Kyn levels in the automated Kyn assay. Cell count was evaluated using Hoechst 33342 for compound's cytotoxicity. Data are mean values ± S.D., n ≥ 3.}
\end{table}
of IDO1. In line with this, BRD4 inhibitors, such as PFI-1, decreased Kyn levels in cells (Table 1). The second group was comprised of small molecules like succinylacetone that interfere with cellular heme synthesis, reduce the abundance of the cofactor and thereby impair IDO1 activity (Table 1). Thus, the cellular Kyn assay successfully detects direct IDO1 inhibitors and can also uncover modulators of cellular Kyn production with different mechanisms of action.

Thiohydantoin inhibitor 6 (IC$_{50}$ = 6.3 ± 2.2 nM), oxazole-4-carboxamide inhibitor 7 (IC$_{50}$ = 4.8 ± 1.2 nM) and piperazin-2-one inhibitor 8 (IC$_{50}$ = 29.8 ± 13 nM; Figure 2A and B) are the most potent compounds identified in the screen and were selected for further characterization (see Figure S4 for more derivatives). An orthogonal, sensor-free method using LC-MS/MS for Kyn detection validated the reduction of Kyn levels upon treatment of BxPC3 cells with 6, 7 or 8 (Figure 2C). In addition, the dose-dependent reduction of Kyn levels by 6, 7 and 8 was detected using p-DMAB in BxPC3 cells and in the human ovarian carcinoma cell line SKOV3 upon stimulation with IFNγ as well as in HEK293T cells that transiently express human IDO1 (Figure S6).

To identify potential direct modulators of IDO1, compounds 6, 7 or 8 (30 μM) were incubated with recombinant human IDO1 protein (rhIDO1) prior to the determination of IDO1 activity. Preincubation of IDO1 with the compounds at 37°C but not at 20°C substantially decreased IDO1 activity (Figure 3A) with IC$_{50}$ values of 0.97 ± 0.59 μM (6), 1.52 ± 1.09 μM (7) and 5.28 ± 0.92 μM (8) (Figure 3B). A similar observation has been made for the heme-competitive IDO1 inhibitor BMS-986205. In cells, heme-competitive IDO1 inhibitors bind to apo-IDO1 and prevent heme binding, holo-IDO1 formation and IDO1 activity. In vitro, holo-IDO1 is used to evaluate IDO1 activity, and inhibitors binding to apo-IDO1 require heme dissociation from holo-IDO1, which in turn requires temperatures of 30°C or higher. At lower temperatures, heme-competitive inhibitors fail to suppress IDO1 activity. However, even at appropriate temperatures, heme dissociation and, thus, formation of apo-IDO1 in vitro is a reversible and slow process. In vitro, the delay of heme dissociation results in a remaining fraction of active holo-IDO1 even in the presence of heme-competitive inhibitors, which reduces the potency of those inhibitors and explains the different IC$_{50}$ values for 6, 7 and 8 in the enzymatic assay compared to the cell-based assay.

For further characterization, we employed differential scanning fluorimetry (DSF) to explore the melting behavior of IDO1.
of IDO1 in the presence of the compounds. Compound 6, 7 and 8 caused a distinct change of the melting curve compared to the DMSO control (Figure 3C), and BMS-986205 induced a similar alteration (Figure 3D). In contrast, epacadostat, which is a Trp-competitive IDO1 inhibitor,[9a] led to thermal stabilization of IDO1 with a shift in melting temperature of $\Delta T_m = 4.1 \pm 1.2 \degree C$ without major change in the melting curve (Figure 3D and S7). To explore whether heme displacement is responsible for IDO1 inhibition by 6, 7 and 8, the spectroscopic properties of IDO1 were analysed in the presence of the compounds. Heme-containing proteins exhibit a characteristic absorbance peak at 405 nm, the so-called Soret band, which corresponds to the electronic state of the iron.[21] A shifted Soret peak indicates a protein-ligand interaction, and a reduced intensity can reveal heme loss. The IDO1 absorption spectrum in the presence of 6, 7 or 8 unveiled a clear, concentration-dependent reduction in Soret band intensities similar to the known heme competitor (Figure 4A). In addition, 14 µM hemin reduced the potency of 6, 7 and 8 to inhibit rhIDO1 activity as detected by an at least 6.5-fold increase in the $IC_{50}$ values to 8.2 ± 1.4 µM (6), 9.7 ± 0.5 µM (7), > 30 µM (8) (Figure 4B). These findings strongly suggest that compound 6, 7 and 8 reduce cellular Kyn levels by competing with heme for apo-IDO1, thereby suppressing IDO1 activity.

In conclusion, we have developed a novel cell-based method to identify IDO1 inhibitors by fluorescence-based monitoring of cellular Kyn levels. The method enables the identification of direct IDO1 inhibitors as well as modulators of IDO1 expression or heme biosynthesis, which are considered indirect IDO1 inhibitors. Three IDO1-inhibitor chemotypes were identified that reduce IDO1 activity by a heme-competitive mechanism. Our findings underscore the power of a cell-based approach to identify IDO1- and Kyn level modulators and to overcome limitations related to biochemical and biophysical assays employing reox-sensitive proteins like IDO1.

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Conflict of interest

The authors declare no conflict of interest.

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