Bioorthogonal Probes for the Study of MDM2-p53 Inhibitors in Cells and Development of High-Content Screening Assays for Drug Discovery

Pier Luca D’Alessandro*, Nicole Buschmann*, Markus Kaufmann, Pascal Furet, Frederic Baysang, Reto Brunner, Andreas Marzinzik, Thomas Vorherr, Therese-Marie Stachyra, Johannes Ottl, Dimitrios E. Lizos,* and Amanda Cobos-Correa*

Abstract: To study the behavior of MDM2-p53 inhibitors in a disease-relevant cellular model, we have developed and validated a set of bioorthogonal probes that can be fluorescently labeled in cells and used in high-content screening assays. By using automated image analysis with single-cell resolution, we could visualize the intracellular target binding of compounds by co-localization and quantify target upregulation upon MDM2-p53 inhibition in an osteosarcoma model. Additionally, we developed a high-throughput assay to quantify target occupancy of non-tagged MDM2-p53 inhibitors by competition and to identify novel chemical matter. This approach could be expanded to other targets for lead discovery applications.

The tumor suppressor protein p53 plays a pivotal role in DNA repair, cell cycle regulation, apoptosis, angiogenesis, and senescence.[1] The p53 protein is mutated or deleted in half of human cancers. In the remaining cases, p53 retains its wild type form, but its activity is inhibited by the human murine double minute 2 (MDM2, also known as HDM2) oncoprotein.[2] Restoration of p53 activity by inhibiting the wild type form, but its activity is inhibited by the human murine double minute 2 (MDM2, also known as HDM2) oncoprotein.[2] Restoration of p53 activity by inhibiting the p53-MDM2 interaction is an appealing therapeutic strategy and senescence.[1] The p53 protein is mutated or deleted in DNA repair, cell cycle regulation, apoptosis, angiogenesis, oncoprotein.[2] Restoration of p53 activity by inhibiting the murine double minute 2 (MDM2, also known as HDM2) wild type form, but its activity is inhibited by the human half of human cancers. In the remaining cases, p53 retains its applications.

To initiate drug discovery activities, there is a need for the development of reliable biological assays, preferably in a relevant disease model. To do that, chemical probes are very useful because they allow the modulation of a target in a biological system.[10] Well validated chemical probes are therefore crucial to generate relevant biological hypotheses, and there is a growing interest in expanding these tools.[6]

Bioorthogonal (BOC) probes can be generated by modifying existing chemical probes with moieties that are inert in biological systems but very reactive towards other bioorthogonal groups. Bioorthogonal reactions can therefore take place within a living system without interfering with biological processes.[7] The requirements for bioorthogonality are good selectivity, fast kinetics, biological and chemical inertness, and biocompatibility.[8] A growing number of reactions fulfill these requirements, allowing the selection of the best chemical transformation for a given application.[9] The appropriate derivatization of the chemical probes is important to minimize the modification of their biological and physico-chemical properties. Contrary to chemical probes, bioorthogonal probes can be modified in cells with reporters such as fluorophores, allowing their direct visualization rather than measuring downstream effects. Some bioorthogonal probes have been developed and successfully used for imaging the behavior of small molecules, such as taxol[10] and PARP1 ligands,[11] or proteins, such as Aurora A, in cells.[12]

Herein, we aimed to develop a potent, selective and well validated bioorthogonal probe based on the potent MDM2-p53 protein–protein-interaction inhibitor class of 3-imidazolylindoles.[13] The properties of these MDM2-p53 inhibitors were studied in a cellular model of osteosarcoma. With this probe, we were able to visualize the differential expression and the subcellular distribution of MDM2 and also to determine the target occupancy of compounds. Additionally, we have designed a cellular high-content imaging assay in a high-throughput format to allow the identification of novel inhibitors of MDM2-p53 interaction using automated image and data analysis.

To find a suitable system for intracellular labeling, we synthesized a set of probes based on 3-imidazolylindoles, a potent MDM2-p53 inhibitor class, containing different tags for bioorthogonal chemistry. Docking of compound 1 in a crystal structure of MDM2 clearly suggested that the introduction of bioorthogonal tags by functionalizing the carboxylic acid would expose the label towards the solvent.

Supporting information, including experimental details, for this article can be found under: http://dx.doi.org/10.1002/anie.201608568.

These are not the final page numbers!
reaction rate was after 4 h under the tested conditions (the calculated average measured by LC-MS. All the reactions reached completion reactivity of different pairs of fluorophores and probes was cells) to guide the design of the cellular experiments. The cells, we determined their reactivity in vitro (in absence of wavelengths for imaging (Supporting Information). for labeling and offered opportunities to study a range of (BCN) as reactive moieties. This approach enabled flexibility functionalized with tetrazine (Tz) or bicyclo[6.1.0]non-4-yne interaction inhibition (see the Supporting Information). 

The potency of the probes towards MDM2 and MDM4 was assessed by TR-FRET. All probes retained inhibitory activity for the MDM2/p53 interaction below nanomolar concentration as for compound 1. Moreover, the functionalization was not detrimental for selectivity against MDM4/p53 interaction inhibition (see the Supporting Information).

As labels, we synthesized several cell permeable fluorophores (8–12), based on BODIPY and TAMRA dyes functionalized with tetrazine (Tz) or bicyclo[6.1.0]non-4-yn-3-ylindole scaffold with strained alkenes (compounds 3–6) or an azide (compound 7). B) Compound 3 docked in MDM2 crystal structure. without interfering with activity. Therefore, this functionality was identified as a viable site for chemical modification (Figure 1 and the Supporting Information, Figure S1). To perform the inverse electron demand Diels–Alder (iEDDA) and strain promoted alkyne-azide cycloaddition (SPAAC) reactions, we modified compound 1 with trans-cyclooctene (TCO) (3), a methyl cyclopropene (4–6), and azide groups (7).

The potency of the probes towards MDM2 and MDM4 was assessed by TR-FRET. All probes retained inhibitory activity for the MDM2/p53 interaction below nanomolar concentration as for compound 1. Moreover, the functionalization was not detrimental for selectivity against MDM4/p53 interaction inhibition (see the Supporting Information).

As labels, we synthesized several cell permeable fluorophores (8–12), based on BODIPY and TAMRA dyes functionalized with tetrazine (Tz) or bicyclo[6.1.0]non-4-yn-3-ylindole scaffold with strained alkenes (compounds 3–6) or an azide (compound 7). B) Compound 3 docked in MDM2 crystal structure.

Accordingly, we discontinued the use of those labels (Supporting Information, Figure S4). For the remaining fluorophores, we determined aggregation by dynamic light scattering (DLS) to choose the appropriate concentration of dye for our experiments. The synthesized fluorophores showed aggregation at concentrations above 5 μM. Thus, we performed the cellular staining experiments with 1 μM of dye (Supporting Information, Figure S5). BODIPY-Fl-Tz (8), BODIPY-TMR-Tz (9), and BODIPY-Fl-BCN (11) dyes have the required properties for our studies: aqueous solubility, cellular permeability, and homogeneous intracellular distribution.

To assess the permeability of the bioBOC probes, we incubated compounds 3–7 at different concentrations with SJSA-1 cells for one hour at 37°C and labeled them with the selected dyes. The reactivity of probes and dyes was tested to find the combinations of BOC pairs that return the highest sensitivity, the fastest kinetics, and lowest background under live and fixed conditions (Figure 2 and Supporting Information, Figure S6).

All probes showed high permeability, accumulating around the nuclei. Several pairs reacted similarly well,
offering high sensitivity for the detection of small molecules in cells and labeling flexibility and allowing the visualization of an increasing concentration of our probes inside cells starting as low as 50 nM.

The permeability of a molecule does not ensure its binding to the target. We aimed to demonstrate the target engagement of our probes by determining their potency with a functional readout and analyzing their subcellular distribution and target-probe co-localization. The disruption of the p53/MDM2 interaction by a small molecule should stabilize p53 and activate the pathway leading to concomitant elevation of the p53 transcriptional target MDM2 in cells with wild-type p53. Therefore, we first assessed the potency of our probes by measuring the overproduction of MDM2 in cells by immunostaining. Thus, we incubated SJSA-1 cells overnight with the probes or with a cell-penetrating peptide (14), which is not known to interact with any intracellular target, as a control. As shown in Figure 3, compounds 1–7 significantly induced MDM2 protein levels with EC50 values in the nanomolar range (Table 1). Compound 1 has a relative potency more than 3-fold lower than compounds 2–7. This is expected because the presence of the carboxylic group in 1 might have a detrimental effect in its cellular permeability. Saturation was reached at 0.5 µM. As expected, the control compound (14) did not show any effect.

Table 1: EC50 of MDM2 induction for different bioorthogonal probes.

<table>
<thead>
<tr>
<th>Probe</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 [nM]</td>
<td>291</td>
<td>100</td>
<td>43</td>
<td>92</td>
<td>42</td>
<td>50</td>
</tr>
</tbody>
</table>

MDM2 was exclusively localized in the nuclei of the cells (Figure 4A). If there is target engagement, the accumulation of MDM2 in nuclei should be followed by probe co-localization. Using the same experimental setup as above, we monitored MDM2 by immunostaining and labeled compounds 3–5 and 7 with different dyes under fixed and live conditions. It was expected that for compounds with an extremely short half-life of dissociation it might be difficult to visualize the co-localization with the target. Therefore, the in vitro kinetic parameters of four probe-target pairs were measured (see the Supporting Information). All probes tested showed a low dissociation rate constant (koff) and a half-life of dissociation (T1/2, dissociation) between 17 and 58 min. Using single-cell data analysis, we could observe a clear correlation between MDM2 levels and probe-labeling intensity in the nucleus verifying that the labeling occurred exclusively in those cells with high levels of MDM2 (Figure 4A,D) thus suggesting probe–target binding. Moreover, since MDM2 levels correlate with labeling, biorthogonal probes can be used to visualize and measure target levels. Whereas all probes localized to cells with nuclear MDM2, the control peptide (14) did not (Figure 4B). Among the BOC pairs, some showed a more pronounced co-localization than others, most probably influenced by the different accessibility of the

Figure 3. Potency of the probes on SJSA-1 quantified as percentage of cells with high levels of MDM2 in nuclei.

Figure 4. Co-localization of probe and MDM2. A) The TCO Probe (3) (160 nm) was incubated overnight with SJSA-1 cells to induce high MDM2 levels in nuclei. After BOC reaction, the probe is co-localized with MDM2 on activated cells. B) The same experiment was done with a non-arginine derivative (14), which does not bind MDM2. Consequently, a lack of MDM2 expression and co-localization are observed. C) The experiment was repeated incubating the probe with HT29 cells, which have a mutated form of p53. The lack of MDM2 overexpression correlates with the absence of the probe in nuclei. D) Single-cell analysis shows a linear correlation between the intensity of probe 3 in the nucleus and MDM2 expression. Scale bar = 50 µm.
probes for labeling, the reaction kinetics, the stability and/or the intracellular dye distribution. Taken together, these results combined with the in vitro assessment and the permeability properties of dyes pointed to BODIPY-FL-Tz–TCO as one of the most appropriate pairs for performing intracellular reactions (Supporting Information, Figure S7). Therefore, we carried out further experiments with this system.

To validate the mode of action of the probes and assess their selectivity towards the MDM2-p53 protein–protein interaction, we performed the target engagement experiment on the HT29 cell line, which unlike SJSA-1, has a mutant form of p53 that prevents DNA binding and, thus, its tumor suppressor function. Since p53 is not active, the inhibition of MDM2-p53 binding will not be counteracted by over-expression of MDM2 and protein levels will remain low. As expected, in this case both the functional and the co-localization readouts showed no MDM2 induction or probe translocation to the nuclei (Figure 4C).

Following validation of our MDM2 probe, we designed a set of assays amenable for lead finding to determine target occupancy of molecules and to identify novel chemical matter. The assays were established in 384-well plates to allow high throughput, automated image analysis, and multi-parametric data analysis at the single-cell level.

Target occupancy of a small molecule is important for predicting its therapeutic efficacy and for validating its mode of action. Several techniques are available to quantify target occupancy in cells. Some of them, such as BRET, require derivatized compounds. The cellular thermal shift assay (CETSA) allows the measurement of the target occupancy of non-labeled molecules engaged with endogenous protein but it is limited to proteins showing thermal stabilization upon ligand binding. To overcome these limitations, we designed a competition binding assay to quantify the amount of endogenous target bound to an untagged MDM2-p53 inhibitor using the biorthogonal TCO probe. This assay relies on the incubation of cells with a fixed concentration of the probe to induce MDM2 overexpression followed by accumulation of the probe in the nuclei. An untagged inhibitor can compete with the probe for binding of the target. Only if there is specific competition, will displacement or reduction of the probe localized to nucleus be observed, otherwise the probe will remain as initially located.

As shown in Figure 3, maximum response is reached with 0.5 μM of the TCO probe (3) and therefore we assume full occupancy of the target at this concentration. We then incubated the system with several concentrations of a set of untagged MDM2-p53 inhibitors (1, 28–31) with different scaffolds and with different cellular potencies to compete out the probe (Figure 5 and the Supporting Information, Figure S8). Using single cell data analysis, we could quantify the reduction of probe with increasing concentrations of inhibitors exclusively in the nuclei of those cells with high levels of MDM2. As expected from the EC50 values of the small molecules (Table 1, Figure S8), 50% of the probe 3 is displaced and, therefore, 50% of the target is occupied by compound 1 at 5 μM (Figure 5C). Target occupancy of compounds 29–31 occurs at higher concentrations than for 1 as expected from their lower potency in cells. Compound 28, a member of the pyrazolopyrrolidinone family is too weak and no occupancy is observed up to 50 μM. In principle, this assay can be used in a screening mode to identify novel compounds binding to MDM2 as potential hits for cancers that overexpress MDM2.

In summary, we have shown how a potent chemical probe can be transformed into a bioorthogonal probe to validate its biological effect in relevant cellular models. The choice of tags and dyes has a great impact on probe reactivity, accessibility of probes in cells, permeability, and stability. Accordingly, an optimization of suitable tools is mandatory before developing further assays. Once developed, biorthogonal probes can be used to establish assays to identify novel chemical matter in a high-throughput setup. Potency and target binding can then be established for each compound using high-content imaging. The methods presented here for MDM2 could be applied to other targets and disease models, therefore opening doors to novel screening assays.

Figure 5. Target occupancy of compound 1. SJSA-1 cells were incubated with 0.5 μM probe 3 to induce high MDM2 levels in nuclei. A) In the absence of competitor 1 there is full co-localization of the probe and target. B) In the presence of 15 μM compound 1 the probe has been competed out. C) Quantification of target occupied as average per well after competing with compounds 1, 28–31. Scale bar = 50 μm.
Acknowledgements

We are very grateful to Dr. Karin Briner, Dr. Peter Fuerst, Prof. Elena Dubikovskaya, Dr. Gregory Hollingworth, Dr. Keiichi Masuya, Dr. Stephan Ruetz, Dr. Kaspar Zimmermann and Dr. Ulrich Schopfer for their constructive suggestions, advice, and support.

Keywords: bioorthogonal chemistry · drug discovery · fluorescent probes · protein–protein interactions · target engagement

Bioorthogonal probes were developed to study MDM2-p53 inhibitors in an osteosarcoma cell model. Target engagement of the probe, target upregulation upon MDM2-p53 inhibition, and target occupancy of non-tagged MDM2-p53 inhibitors could be monitored by using high-content screening assays and automated single-cell imaging analysis.