Molecular Rotors As Conditionally Fluorescent Labels for Rapid Detection of Biomolecular Interactions

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S *Supporting Information*

ABSTRACT: We demonstrate the use of fluorescent molecular rotors as probes for detecting biomolecular interactions, specifically peptide−protein interactions. Molecular rotors undergo twisted intramolecular charge transfer upon irradiation, relax via the nonradiative torsional relaxation pathway, and have been typically used as viscosity probes. Their utility as a tool for detecting specific biomolecular interactions has not been explored. Using the well characterized p53−Mdm2 interaction as a model system, we designed a 9-(2-carboxy-2-cyanovinyl) julolidine-based p53 peptide reporter, JP1-R, which fluoresces conditionally only upon Mdm2 binding. The reporter was used in a rapid, homogeneous assay to screen a fragment library for antagonists of the p53−Mdm2 interaction, and several inhibitors were identified. Subsequent validation of these hits using established secondary assays suggests increased sensitivity afforded by JP1-R. The fluorescence of molecular rotors contingent upon target binding makes them a versatile tool for detecting specific biomolecular interactions.

Molecular rotors are an emerging class of fluorophores[1] characterized by their ability to undergo twisted intramolecular charge transfer (TICT).[2] They typically consist of three parts: an electron-donating unit, an electron-accepting unit, and a π-conjugated linking moiety, which allows electron transfer to occur in the planar conformation. However, electrostatic forces upon irradiation result in the molecule adopting a twisted conformation around the σ-bond in the linker region. This nonplanar, twisted conformation has a lower excited-state energy and is associated with either a red-shifted fluorescence emission or can undergo a nonradiative torsional relaxation pathway, depending on the molecular structure of the rotor.[3] This TICT property of molecular rotors is highly sensitive to their environment.[4] If the intramolecular rotation is hindered, through higher viscosity for instance, the nonradiative pathway is prevented, and the molecule relaxes via the radiative pathway, thus restoring fluorescence. The inherent sensitivity of molecular rotors to the polarity and viscosity of the environment has fuelled their application as viscosity sensor probes.[5] Their fluorescence lifetime has also been used as a quantitative parameter for ratiometric measurement of the viscosity in live cells.[6]

Although molecular rotors such as acridiziniums and thioflavin-T have been shown to bind to albumin proteins[7] and amyloid fibrils,[8] their use in detecting specific biomolecular interactions has yet to be explored.[3] In contrast, another class of environment-sensitive fluorophores, solvatochromic compounds, have been highly useful in a plethora of research applications,[9] and probes incorporating solvatochromic fluorophores for detecting specific protein interactions have also been reported.[10] These compounds exhibit changes in fluorescence emission and quantum yield due to changes in the polarity and hydration of their environment. Here, we investigate the environment-dependent fluorescence of molecular rotors in the context of specific biomolecular interactions.

We first conjugated the molecular rotor, 9-(2-carboxy-2-cyanovinyl) julolidine (CCVJ), to biotin and measured fluorescence changes upon binding to streptavidin. A 2-fold fluorescence increase was measured upon the addition of streptavidin, demonstrating the utility of the biotin-rotor probe (Figure S1). The behavior of molecular rotors when coupled to interacting proteins was next investigated. For our model system, we chose the interaction between Mdm2 and p53, a clinically relevant interaction and important target in drug discovery.[11] We conjugated the CCVJ rotor to two peptides derived from a phage display screen, JP1 and JP2, which differ by only a single amino acid but have a 10-fold difference in binding affinities[12] (Table 1). An additional lysine residue was added to the C-termini of both peptide sequences and then reacted with the *N*-hydroxysuccinimidyl-activated ester of CCVJ to make the rotor-peptide probes (Scheme 1).

Affinity-purified recombinant Mdm2 protein (residues 18−125) was used to test the functionality of the rotor-peptide

Table 1. Amino Acid Sequence and Dissociation Constants of Peptide Variants Used in This Study

peptide ID	amino acid sequence	K_d (nM)[a]
JP1	MPRFMDYWEGLSK	18.83 ± 5.03
JP2	MPRFMDYWEGLNK	239.81 ± 53.79

[a]Without lysine residue at C-terminus[12]

Scheme 1. Synthetic Scheme for Rotor−Peptide Conjugates
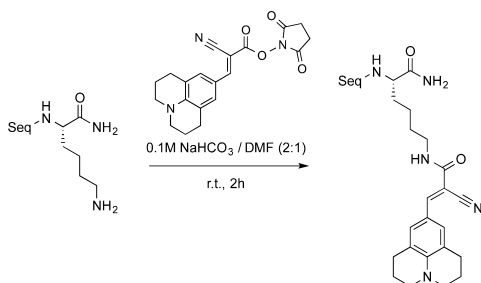
probes. Co-incubation of JP1-rotor conjugate, JP1-R, with Mdm2 protein, led to a concentration-dependent increase in fluorescence activity (Figure 1A). Nutlin-3, an Mdm2 antagonist, binds Mdm2 at the N-terminal hydrophobic cleft and abrogates this interaction by occluding p53.[13] Addition of racemic Nutlin to the JP1-R−Mdm2 complex completely abrogated the fluorescence signal seen before (Figure 1B), presumably due to displacement of JP1-R from Mdm2. JP2-R did not display any significant changes in fluorescence intensity upon adding Mdm2. We ascribed this lack of signal to the nonconstrained orientation of the rotor upon binding of the peptide to Mdm2 (see below).

To further demonstrate that fluorescence activation was due to a concomitant steric restriction of the appended rotor during protein-specific interaction, we added JP1-R to nonspecific proteins eIF4E, BSA, and IgG. No fluorescence increase was observed with all 3 proteins across the same concentrations range (Figure 1C). Additionally, the specificity conferred by the peptide sequence was critical for JP1-R's binding-induced fluorescence, as nonconjugated molecular rotor did not result in any fluorescence increase when added to Mdm2 (Figure S3). Furthermore, the low levels of rotor activity in the presence of nonspecific proteins indicate potential use in complex biological samples.

Based on the JP1-R fluorescence measurements (Figure S2), we calculated an apparent K_d of 16.01 ± 7.52 nM for Mdm2 binding (Figure S4), correlating well to a previously reported value of 18.83 ± 5.03 nM (Table 1) determined using isothermal titration calorimetry (ITC).[12] To further validate the binding of JP1-R, we performed ITC experiments and obtained a K_d of 10.2 ± 7.97 nM for JP1-R, consistent with the value obtained via fluorescence (Figure S5). The calculated apparent K_d of JP2-R was 3365 ± 640.6 nM, ~14-fold lower than the previously reported value of 239.81 ± 53.79 nM.

To further understand the fluorescence-derived apparent dissociation constants of JP1-R and JP2-R, we performed an in silico modeling of their respective interactions with the Mdm2 protein. Molecular dynamics simulations suggest that the C-terminal end of JP1-R adopts a helical turn due to the constraints from the hydrogen bonds between the hydroxyl side chain and backbone of S12 and the backbone carbonyl of W8. A similar feature was also seen in prior experimental and
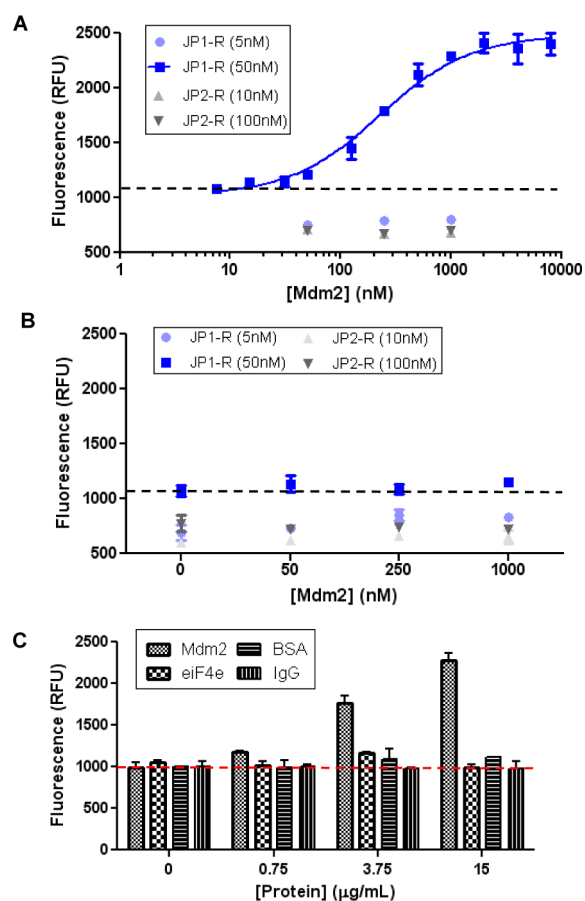
Figure 1. Fluorescence of JP1-R increases when bound specifically to recombinant Mdm2 (residues 18−125). (A) Increasing concentrations of Mdm2 were added to fixed concentrations of rotor-peptide conjugates, JP1-R and JP2-R. (B) Fluorescence from binding between Mdm2 and JP1-R was ablated with the addition of Nutlin (50 μM). (C) JP1-R (50 nM) was added to increasing amounts of Mdm2 (residues 18−125), eiF4e, BSA, and IgG. Hashed-line shows background fluorescence from JP1-R only (50nM). Error shows average ± SD (n = 3).

computational studies for a similar peptide.[14] The replacement of S12 by N12 in JP2-R does not afford this constraint. The Asn side chain is longer and is unable to form hydrogen bonds with the backbone, resulting in an extended C-terminus (Figure 2, videos 1 and 2). Replica exchange simulations exploring the conformational space of the unbound rotor-peptides (Table S1) show that JP1-R is more helical than JP2-R. The constrained JP1-R (Figure 2) also embeds deeper into
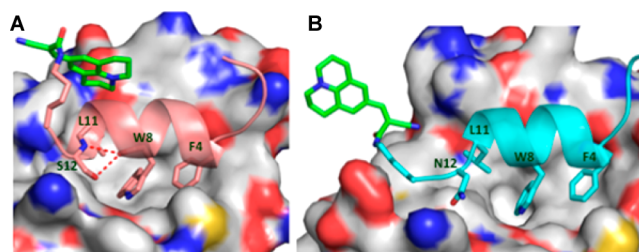
Figure 2. In silico model of rotor-peptide conjugates (A) JP1-R and (B) JP2-R bound to Mdm2 pocket. Hydrogen-bond interactions of W8 backbone with S12 backbone and side chain are shown in red dashed lines. Rotor moiety depicted in green.

Mdm2 and interacts stronger than JP2-R (by ~7 kT, Table S2). The major contribution arises from improved packing of S12 (by 1.2kT, Table S3a,b), L11 (embeds deeper into Mdm2 by ~2 kT), and the rotor (~2kT). The rotor packs between H96 of Mdm2 and the peptide in JP1-R, while in JP2-R, it packs into a recently characterized second binding site in Mdm2.[15] This tighter association of JP1-R restricts the rotational freedom of the rotor sufficiently to bring about a detectable fluorescence turn-on signal.

The p53 protein is a critical tumor suppressor found mutated in >50% of all human cancers[16] and is primarily regulated by the ubiquitin E3 ligase, Mdm2, which targets it for proteasomal degradation. Inhibiting the Mdm2-p53 interaction represents an attractive therapeutic modality, especially in human cancers carrying wild-type p53.[17] The primary interaction between Mdm2 and p53 has been mapped to the N-terminal of both p53 (residues 18−26) and Mdm2 (residues 1−110).[18] Several compounds that bind to the N-terminal domain of Mdm2 and abrogate p53 binding have shown promise in preclinical development.[13] High-throughput screens for novel compounds will thus benefit greatly from robust, facile, and sensitive methods enabling detection of the p53-Mdm2 interaction.[19]

To demonstrate the utility of the rotor-peptide probe in small molecule drug screening, we first explored the sensitivity of the rotor-peptide to known small molecule and stapled-peptide inhibitors of the p53-Mdm2 interaction.[20] These inhibitors target the same hydrophobic cleft in Mdm2 as JP1-R and were able to disrupt the Mdm2-probe complex in the expected manner, resulting in a decrease in fluorescence as the probe was displaced (Figure 3). Moreover, the dissociation constant of each inhibitor derived from using JP1-R corresponded well to previously reported values (Figures S6 and S7).

Given the high sensitivity and specificity of the JP1-R conjugate, we next used it to screen a small molecule fragment library (n = 352) for candidates that potentially disrupt p53-Mdm2 binding. Based on the results, 15 hits were
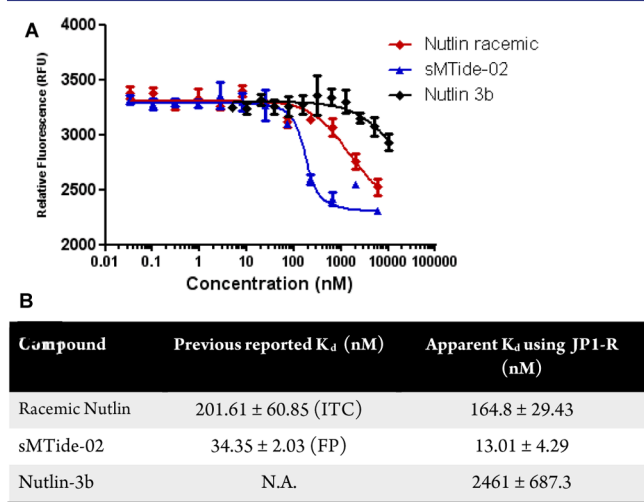

Figure 3. JP1-R reporter is sensitive to several known inhibitors of the p53-Mdm2 interaction. (A) Titration of known p53-Mdm2 inhibitors onto prebound Mdm2-JP1-R reactions. Racemic Nutlin consists of 3a + 3b, of which Nutlin 3a is the more active enantiomer. (B) Calculated K_ds using JP1-R of compound inhibitors correlated well with previous reports of K_d.

Compound	Previous reported K_d (nM)	Apparent K_d using JP1-R (nM)
Racemic Nutlin	201.61 ± 60.85 (ITC)	164.8 ± 29.43
sMTide-02	34.35 ± 2.03 (FP)	13.01 ± 4.29
Nutlin-3b	N.A.	2461 ± 687.3

selected for further validation (Figures 4A, S8) using fluorescence polarization (FP),[21] and 8 compounds were
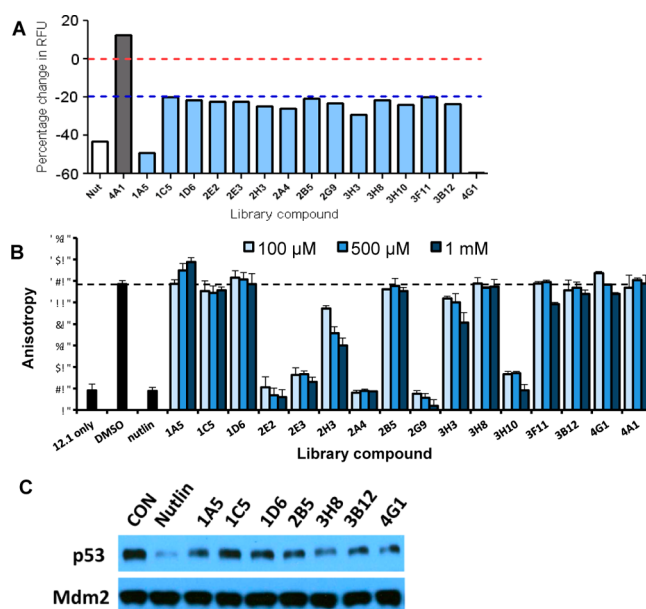

Figure 4. Fragment library screen and lead validation for p53-Mdm2 inhibition. (A) Positive lead compounds (blue bars) from library screen using JP1-R. White and gray bars represent positive control (1 μM Nutlin) and inactive negative control (500 μM 4A1), respectively. Red hashed line indicates reference fluorescence level (JP1-R bound to Mdm2), below which indicates a positive displacement event. Blue hashed line indicates designated threshold level for a positive fragment hit. (B) 8 out of 15 hits were validated by fluorescence polarization assay, showing ligand-dependent (100 μM, 500 μM or 1 mM) displacement. Black bars depict control measurements of FAM-labeled 12.1 peptide, DMSO negative control, and 50 μM Nutlin positive control. Inactive compound 4A1 shows negative displacement control. Error shows SD of triplicate measurements. (C) Weaker inhibitor fragments were further assessed through their ability to displace in vitro translated full-length p53 protein bound to recombinant Mdm2-immobilized on cobalt beads. Western blot shows levels of p53 (upper panel) captured in the presence of indicated compounds (500 μM). Lane 1 is the negative control (inactive 4A1 fragment), and lane 2 is the positive control (100 μM Nutlin). Lower panel indicates input Mdm2 levels eluted off beads.

further confirmed as genuine inhibitors (Figure 4B). The seven disparate compounds showing no activity in the FP assay[22] were therefore assessed in a pull-down assay[22] which measured the direct interaction levels of p53 and Mdm2. All seven compounds inhibited p53-Mdm2 interaction, further validating these as genuine hits not identified by the FP assay (Figure 4C). As subtle intermolecular twisting of the rotor profoundly effects signal generation (Figure 2), it is possible that partial displacement of the peptide by the weak inhibitors led to their identification. In the FP assay, these would have been missed, as the anisotropy measurement is largely attendent on full displacement of peptide from Mdm2.

Together, these results demonstrate the utility of molecular rotors in binding assays for detecting peptide−protein interactions and for drug screening applications.[19] Using the TICT property of the molecular rotor, its free volume is decreased upon binding interaction with a protein. This simple fluorescence turn-on signal upon protein binding

allows the development of highly sensitive and facile assays to measure protein–ligand binding in a high-throughput fashion. More importantly, we have shown that a molecular rotor-based screening assay identified validated hits that were missed by fluorescence polarization assay in a fragment-based screen, suggesting its utility in identifying lower affinity hits in fragment-based screening.[23]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic details and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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