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Ligand-directed protein labelling can be used to introduce diverse chemical functionalities onto proteins without the need for incorporation of genetically encoded tags. Here we report a method for the rapid and efficient labelling of a protein using a ruthenium-bipyridyl (Ru(II)(bpy)3) modified peptide designed to mimic an interacting BH3 ligand within a BCL-2 family protein-protein interaction (PPI). Using sub-stoichiometric quantities of (Ru(II)(bpy)3)-modified NOXA-B and irradiation with visible light for 1 minute, the anti-apoptotic protein MCL-1 was photolabelled in a ligand-dependent manner with a variety of functional tags, as determined by in-gel fluorescence, affinity purification, and ESIMS analysis. In contrast with previous reports on Ru(II)(bpy)3-catalysed photolabelling, tandem MS experiments revealed that the dominant labelling occurred on a cysteine residue of MCL-1. Labelling of MCL-1 occurred selectively in mixtures with other proteins, including the structurally related BCL-2 member, BCL-xL. These results improve methodology for proximity-induced photolabelling of proteins, demonstrate the approach is applicable to interfaces that mediate PPIs, and pave the way towards future use of ligand-directed proximity labelling for dynamic analysis of the localisation and interactome of BCL-2 family proteins.
Photocatalytic proximity labelling of MCL-1 by a BH3 ligand

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Abstract

Ligand-directed protein labelling can be used to introduce diverse chemical functionalities onto proteins without the need for incorporation of genetically encoded tags. Here we report a method for the rapid and efficient labelling of a protein using a ruthenium-bipyridyl (Ru(II)(bpy)₃) modified peptide designed to mimic an interacting BH3 ligand within a BCL-2 family protein-protein interaction (PPI). Using sub-stoichiometric quantities of (Ru(II)(bpy)₃)-modified NOXA-B and irradiation with visible light for 1 minute, the anti-apoptotic protein MCL-1 was photolabelled in a ligand-dependent manner with a variety of functional tags, as determined by in-gel fluorescence, affinity purification, and ESI-MS analysis. In contrast with previous reports on Ru(II)(bpy)₃-catalysed photolabelling, tandem MS experiments revealed that the dominant labelling occurred on a cysteine residue of MCL-1. Labelling of MCL-1 occurred selectively in mixtures with other proteins, including the structurally related BCL-2 member, BCL-xL. These results improve methodology for proximity-induced photolabelling of proteins, demonstrate the approach is applicable to interfaces that mediate PPIs, and pave the way towards future use of ligand-directed proximity labelling for dynamic analysis of the localisation and interactome of BCL-2 family proteins.

Introduction

Most cellular proteins function as dynamic complexes with other proteins and, conversely, protein-protein interactions (PPIs) play key roles in the regulation of most biological processes.1 While stable PPIs are usually associated with multi-subunit protein complexes and quaternary structure, transient PPIs regulate multiple cellular processes, and are implicated in a variety of disease states.2 Ongoing efforts to study transient PPIs may lead to better understanding of the processes of life and the development of novel diagnostics and therapeutics. As part of such efforts, the ability to selectively introduce chemical labels onto proteins involved in specific PPIs would provide new tools to study such PPIs, including the construction of protein-based biosensors or affinity enrichment reagents for dynamic interactome analysis.

Although numerous methods for chemical protein labelling exist, only few are suitable for the selective labelling of (subsets of) native proteins in complex biological samples, such as cell lysates, whole cells and tissues.3 Many of these methods rely on enzymatic activity for the labelling of highly nucleophilic active site residues4 or on the metabolic incorporation of non-natural substrates, such as amino acids,5,6 carbohydrates7 and lipids,8,9 which can be further derivatised through bio-orthogonal chemistry.5,10,11 An effective strategy that does not rely on enzymatic activity or post-translational modifications, and would therefore be especially suitable for the study of PPIs, is the use of ligand-directed protein labelling (LDL).12,13 LDL relies on reagents consisting of a ligand for the protein-of-interest attached to a (moderately) reactive chemical group (exchange/cleavage approach) or to a
catalyst that activates a third component (catalyst tethering approach). Upon binding to its target protein, these LDL reagents cause the transfer of chemical labels to specific, proximal amino acid residues. Hamachi and co-workers have developed a wide range of LDL methods capable of selectively modifying native proteins in a ‘traceless’ manner, in which the ligand leaves its binding site after the labelling reaction and the protein is able to perform its native function. These methods include exchange/cleavage approaches based on electrophilic phenylsulfonate esters, acyl imidazoles, N-sulfonyl pyridines, or N-acyl-N-alkyl sulfonamides and catalyst tethering approaches based on N,N-dimethylaminopyridine (DMAP) or oxime reagents. In addition, several groups have developed LDL reagents incorporating transition metal catalysts. Ball and co-workers used rhodium(II) metallopeptides to selectively modify side-chains on protein surfaces with functionalised diazo compounds. Based on chemistry established by Kodadek et al., the group of Nakamura developed a photocatalytic LDL method based on local single-electron transfer (SET) mediated by a Ru(II)(bpy) submolecule anticancer target in its own right. Irradiation of the ruthenium complex with visible light results in an excited state [Ru(II)(bpy)]\(^6\) complex of relatively long lifetime (~1 ms) that can function as electron donor or electron acceptor. In photocatalytic LDL, the excited [Ru(II)(bpy)]\(^6\) moiety loses an electron to a sacrificial oxidant such as ammonium persulfate (APS) or molecular oxygen, and then catalyses reactions between tyrosine residues proximal to the ligand-binding site and electron-rich dimethylaniline or 1-methyl-4-aryl-urazole (MAUl) derivatives that may act as radical trapping agents (RTAs; e.g., carrying fluorescent or biotinylated labels; Fig. 1a). Photocatalytic LDL reagents based on the ligands benzenesulfonamide, gefitinib and methotrexate were used to label or immobilise carbonic anhydrase II (CAII), epidermal growth factor receptor (EGFR) or dihydrofolate reductase (DHFR), respectively. However, it should be noted that excesses of the ligand-directed Ru(II)(bpy)\(^{3+}\) catalyst were employed in the aforementioned studies, meaning the catalytic potential of this labelling chemistry has not yet been demonstrated.

The majority of LDL approaches are based on interactions of proteins with small-molecule ligands. In contrast, the scope of LDL within the context of PPIs is less well developed, and only few examples use peptides as the ligand component of LDL reagents. Given a significant proportion of PPIs are mediated by peptide interacting motifs, we sought to exploit peptides for the development of LDL reagents for selective labelling of proteins involved in transient PPIs. To enable future studies of PPIs that may require temporal control in the identification, visualisation or perturbation of transient PPIs in future studies, we chose to base our LDL approach on [Ru(II)(bpy)]\(^{3+}\)-mediated photocatalysis.

As a model system, we chose proteins of the B-cell lymphoma 2 (BCL-2) family. BCL-2 proteins regulate apoptotic cell death in response to pro- and anti-apoptotic signals through a variety of transient PPIs between pro-apoptotic (e.g., BAK, BAX), anti-apoptotic (e.g., BCL-2, BCL-xL, MCL-1) and effector (e.g., BID, BIM, PUMA, NOXA-B) members of the BCL-2 family. PPIs in this family are mediated by the binding of a BH3 domain – in a helical conformation – of effector/pro-apoptotic BCL-2 family members to a groove on the surface of anti-apoptotic partners. The exact mechanism(s) via which apoptosis is regulated by PPIs of the BCL-2 family are not yet fully understood, but anti-apoptotic members are over-expressed in certain cancers. Notably, MCL-1 has come into focus as a small-molecule anticancer target in its own right, due to its role in resistance to approved anticancer therapies. To facilitate future studies of BCL-2 family PPIs, in this work our objective was to develop the underlying methodology for selective chemical labelling of native (unmodified) BCL-2 family proteins. Here, we report the rapid and selective photolabelling of MCL-1 using a NOXA-B BH3 peptide incorporating an N-terminal Ru(II)(bpy)\(^{3+}\) substituent (Fig. 1b).
Figure 1: Ligand-directed protein labelling using catalytic Ru(II)(bpy)$_3$ reagents. (a) Previous work: use of small-molecule ligands to promote selective target protein labelling via generation and trapping of tyrosyl radicals. (b) This work: use of a NOXA-B BH3 peptide as the recognition element (ligand), mimicking an interacting partner within the MCL-1/NOXA-B PPI and facilitating SET-mediated ligand-directed MCI-1 labelling.

Results

Design and synthesis of reagents

The BH3 domain NOXA-B$_{75-93(C75A)}$ modified with an N-terminal FITC group (FITC-Ahx-AQLRRIGDKVLNRQKLNNCONH$_2$; FITC-NOXA-B) retains its affinity for MCL-1, as determined by fluorescence anisotropy experiments. Therefore, we designed an LDL reagent for the labelling of MCL-1 consisting of the same BH3 sequence linked to a Ru(II)(bpy)$_3$ photocatalyst via an aminohexanoic acid (Ahx) linker on its N-terminus (Ru(II)(bpy)$_3$-NOXA-B 1; Fig. 2a). LDL reagent 1 was prepared through solid-phase peptide synthesis (Scheme S1-S2) and purified by preparative HPLC (Fig S1). Recombinant MCL-1$_{172-327}$ was expressed and purified as published previously.

Fluorescent (TAMRA-RTA 2) and biotinylated (biotin-RTA 3) radical trapping agents analogous to those developed by Nakamura and co-workers were synthesised through adaptation of literature procedures (Fig. 2a, Scheme S3).

Photocatalytic labelling of MCL-1

For photocatalytic LDL, Ru(II)(bpy)$_3$-NOXA-B 1, TAMRA-RTA 2 and ammonium persulfate (APS) were added to a buffered solution of MCL-1 and the mixture was irradiated for 1 minute at 450 nm using blue LED lamps (see Supporting Information for details). An aqueous solution of the radical scavenger dithiothreitol (DTT, 10 mM) was then added to quench the reaction. Optimisation of the...
labelling conditions resulted in fluorescent modification of MCL-1 using 20 mol% of peptide-catalyst 1 and an equimolar concentration (relative to [MCL-1]) of TAMRA-RTA 2 (lane 1, Fig. 2c). Analysis of the labelled mixture using in-gel fluorescence indicated that ruthenium-modified peptide 1, fluorescent RTA 2 and visible light irradiation were all necessary for efficient labelling of MCL-1. A small amount of background labelling of MCL-1 occurred in the absence of 1, possibly due to the ability of the rhodamine dye in 2 to act as a photoredox catalyst\(^{45,46}\) (lane 3, Fig. 2c; more clearly seen when larger amounts of protein were loaded onto the SDS-PAGE gel: Fig. S10-S12). This explanation is consistent with the absence of background labelling when biotin-RTA 3 was used instead of TAMRA-RTA 2 (see below and Fig. S17). Furthermore, the addition of APS to the reaction mixture was necessary for efficient labelling of MCL-1 in these experiments, despite a small amount of labelling occurring when APS was omitted (lane 4, Fig. 2c). In contrast to previous reports,\(^ {32,34,35}\) a sub-stoichiometric amount of the Ru(II)(bpy)\(_3\) reagent (20 mol% relative to [MCL-1]) was used in these experiments, demonstrating the catalytic potential of this chemistry for protein labelling.

The identity of the labelled species was confirmed by intact protein electrospray ionisation mass spectrometry (ESI-MS). Prior to irradiation, only unmodified MCL-1 (17737 Da, Fig. 2d, left) could be detected in the reaction mixture. However, upon irradiation with visible light for 1 minute, the mass spectrum of the reaction mixture showed peaks corresponding to both unmodified MCL-1 (17737 Da) and the labelled species [(MCL-1) + 2] (18396 Da) (Fig. 2d, left). These data confirm the main reaction product results from the addition of one TAMRA-RTA label, suggesting labelling of a single amino acid residue on MCL-1. Several additional peaks were evident in the MS trace of the irradiated sample (denoted by a star, Fig. 2d), most likely resulting from the oxidation of amino acid residues on MCL-1 proximal to the Ru(II)(bpy)\(_3\) complex (indicated by several mass increases of +16 Da, see Fig. S19). Oxidation of proteins using Ru(II)(bpy)\(_3\) reagents in the absence of a ‘radical trapper’ has been reported previously,\(^ {30}\) and this has been exploited in chromophore-assisted light inactivation (CALI)\(^ {47,48}\) reagents that allow targeted protein inactivation with visible light, \textit{in vitro} and in cells.\(^ {34,36,49,50}\)

Next, we investigated the labelling of MCL-1 using a different functional tag, biotin-RTA 3. ESI-MS analysis of the crude reaction mixture revealed the appearance of a distinct species (18209 Da, Fig. 2d, right) suggesting single biotin labelling of MCL-1 (for control experiments in the absence of individual reagents/conditions, see Fig S17). The biotinylated MCL-1 could be affinity-purified with avidin-agarose beads, demonstrating that the biotin itself had not been oxidised and was accessible (Fig. S18). Although the ESI-mass spectrum appears cleaner than that of TAMRA-labelled MCL-1, irradiation times greater than 1 minute resulted in higher conversion to oxidised protein species – of both unmodified and modified MCL-1 (Fig. S20). Therefore, irradiation times should be kept to a maximum of 1 minute to prevent oxidative damage of the labelled protein, and may need optimisation for specific protein–reagent pairs, sample type and light source.
Figure 2. Labelling of MCL-1 with fluorescent and biotinylated Radical Trapping Agents (RTAs) 2 and 3, mediated by Ru(II)(bpy)$_3$-NOXA-B 1. (a) Chemical structures of ligand-directed labelling reagent Ru(II)(bpy)$_3$-NOXA-B 1, fluorescent label TAMRA-RTA 2 and biotinylated label biotin-RTA 3. (b) Top: Fluorescence anisotropy competition experiment for the inhibition of the FITC-NOXA-B/MCL-1 interaction by Ru(II)(bpy)$_3$-NOXA-B 1 (red) and Ac-NOXA-B 4 (black). Error bars represent the standard deviation of three repeats. Bottom: IC$_{50}$ values for peptides 1 and 4 obtained from fluorescence anisotropy competition assays (± SEM). (c) Fluorescence image (FL) and Coomassie Brilliant Blue (CBB) stained SDS-PAGE gels of photolabelled mixtures; fluorescence image shows labelling of MCL-1 using 1 min irradiation, 5 µM TAMRA-RTA 2, 1 µM Ru(II)(bpy)$_3$-NOXA-B 1 and 10 µM APS; CBB stain shows MCL-1 for all conditions. Reactions were carried out on 5 µM MCL-1 in ammonium bicarbonate buffer, pH 7.4. Pictures of complete gels are shown in the Supporting Information. (d) Deconvoluted ESI-MS spectra of photolabelling reaction mixtures displaying fluorescently modified (18396 Da) and biotinylated MCL-1 (18209 Da). Star denotes possible oxidised protein species (see Fig. S19 for annotated spectrum).

Competition with Ac-BID confirms ligand-directed nature of MCL-1 labelling

To confirm that labelling of MCL-1 was indeed mediated by peptide binding, bringing the ruthenium complex into proximity of the amino acid(s) residue to be labelled, competition experiments were undertaken. Initially, the wild-type sequence, Ac-NOXA-B 4, was used as the competitor peptide in these experiments. However, excess Ac-NOXA-B did not completely abrogate labelling, presumably due to the 10-fold lower inhibitory potency of peptide 4 (2068 ± 334 nM, Fig. 2b) compared with ruthenium(II)-modified peptide 1 (201 ± 14 nM; Fig. 2b). Therefore, another peptide that binds in the same hydrophobic groove of MCL-1 as NOXA-B, but with a comparable IC$_{50}$ value to that of 1 (Ac-BID 5, IC$_{50}$ 390 ± 80 nM) was chosen. Increasing concentrations (0-1000 µM) of Ac-BID 5 were added to the reaction mixture prior to irradiation, resulting in decreasing amounts of fluorescently modified protein – with labelling completely abolished using 1000 µM Ac-BID 5 (Fig. 3a). The concentration of 5 needed to fully suppress labelling may seem high compared to its IC$_{50}$, but is not unreasonable because the labelling is a kinetically controlled, irreversible process, which will therefore depend on labelling time as well as relative affinities of peptide:MCL-1 complexes. The intensity of the fluorescent and Coomassie-stained bands was quantified using ImageJ software, and the data from three
independent repeats were plotted against the concentration of competitor peptide 5, revealing a dose-dependent inhibition of labelling (Fig. 3b). These data confirm that the photocatalytic labelling of MCL-1 is dependent on a specific binding event between MCL-1 and Ru(II)(bpy)₃-NOXA-B 1.

**Figure 3:** Competition experiments to confirm ligand-directed nature of labelling. (a) SDS-PAGE gel showing that fluorescent labelling of MCL-1 mediated by peptide 1 is suppressed by increasing concentrations of Ac-BID (lanes 2-4). Conditions: 5 µM MCL-1, 1 µM 1, 5 µM RTA 2, 10 µM APS, 0-1000 µM Ac-BID 5, 1 min irradiation, 50 mM (NH₄)HCO₃ (pH 7.4). Pictures of complete gels are shown in the Supporting Information. (b) Plot of relative fluorescence intensity for the competition experiment with different concentrations of Ac-BID. Error bars represent the standard deviations from 3 independent repeats.

**MS/MS analysis to determine site of labelling on MCL-1**

Tandem mass spectrometry was used to identify the amino acid residues on MCL-1 that were modified upon Ru(II)(bpy)₃-NOXA-B 1-mediated photolabelling. Minimal RTA 6 (Fig. 4a) was chosen over fluorescent and biotinylated RTAs 2 and 3, due to the relative simplicity of the chemical structure, avoiding complication of MS/MS spectra due to fragmentation of the label in the mass spectrometer. For the photolabelling experiment, 10 µM RTA 6 was used to maximise conversion to the labelled species. Intact protein mass spectrometry (ESI-MS) of the labelling mixture prior to proteolytic digestion confirmed a high conversion in the labelling reaction, and incorporation of a single RTA label 6 (Fig. 4b), consistent with reactions with other RTAs 2 and 3 described above.

Limited proteolysis of MCL-1 before and after modification with RTA 6, using trypsin and Glu-C, resulted in peptide fragments that were analysed using reverse-phase HPLC and Q-TOF MS/MS (Fig. S21-S22; sequence coverage: 97% for unmodified MCL-1 and 94% for modified MCL-1). Peptide mapping analysis identified Cys286 as the only significant site on MCL-1 modified with label 6 (Fig. 4c, Fig. S23), despite the presence of two tyrosine residues in the MCL-1 protein sequence (Tyr175 and Tyr185).
Figure 4: Photolabelling of MCL-1 with RTA 6 to identify the site of modification using tandem mass spectrometry (MS/MS). (a) ESI-MS spectrum of irradiated mixture: 5 µM MCL-1, 10 µM RTA 6, 1 µM peptide 1, 10 µM APS, 1 min irradiation, 50 mM (NH₄)HCO₃, pH 7.4, showing unmodified MCL-1 (17737 Da) and modified MCL-1 (17914 Da). (b) Q-TOF MS/MS spectrum for a selected peptide modified with RTA 6. Observed y and b ions are shown in red and blue, respectively. (c) Mapping of labelled cysteine residue Cys286 (and unmodified tyrosine residues Tyr175 and Tyr185) onto MCL-1/NOXA-B structure (PDB:2NLA). MCL-1 is shown in green; NOXA-B peptide is shown in cyan. The star at the N-terminus of the NOXA-B peptide denotes the position of attachment of linker and Ru(II)(bpy)₃ complex.

MCL-1 is selectively labelled over BCL-2L in a mixture of proteins

Finally, we determined whether Ru(II)(bpy)₃-modified peptide 1 could selectively label MCL-1 over a structurally related BCL-2 family member to which 1 does not bind.⁴⁴ According to fluorescence anisotropy experiments, Ru(II)(bpy)₃-NOXA-B 1 does not inhibit the interaction of BCL-xL (1-198, Δ27-82) with the fluorescently labelled peptide FITC-BID 7 (in comparison to an IC₅₀ value of 201 ± 14 nM obtained for the MCL-1/FITC-NOXA-B interaction; Fig. 5a). Therefore, we evaluated the selectivity of ligand-directed photolabelling with 1 between MCL-1 and BCL-xL. Indeed, in an equimolar mixture of MCL-1 and BCL-xL, MCL-1 was labelled selectively over BCL-xL (lane 1, Fig. 5b). The experiments suggests that a trace amount of BCL-xL can be labelled, but only when MCL-1 is present as well (compare lanes 1 and 5, Fig. 5b). This observation is not completely understood, but there is tentative evidence for an interaction between MCL-1 and BCL-xL.⁵¹ Higher concentrations of TAMRA label 2 (500 µM) led to more non-specific labelling of BCL-xL (Fig. S13), which is consistent with the ability of TAMRA itself to act as photocatalyst.⁴⁵,⁴⁶ MCL-1 was also selectively labelled in a stoichiometric mixture of MCL-1 and hDM2, a regulator of the p53 tumour suppressor (Fig. S14). These results suggest a ligand-directed mode of labelling, i.e. binding of Ru(II)(bpy)₃-NOXA-B to MCL-1 facilitates selective labelling of MCL-1 over proteins it does not bind to.
We have demonstrated the use of the peptide-based protein mimic Ru(II)(bpy)₃-NOXA-B 1 as an LDL reagent for the selective photocatalytic, ligand-directed labelling of the anti-apoptotic BCL-2 protein, MCL-1. SET photolabelling of recombinant MCL-1 with three different dimethylaniline derivatives was achieved, with irradiation times of 1 min, and relative quantification of labelling was achieved using in-gel fluorescence measurements and ESI-MS analysis. This work builds on previous literature describing the use of small molecule-based Ru(II)(bpy)₃-based LDL reagents for the labelling of the enzymes CAII, EGFR and DHFR,³²-³⁴ and demonstrates the catalytic potential of the SET-based LDL technology as well as the suitability of peptide-based LDL reagents for application on interfaces of PPIs. The selective modification of MCL-1 over BCL- xl and hDM2, and the inhibition of MCL-1 labelling by the addition of competitor peptide Ac-BID 5, suggest a ligand-directed mode of labelling. It should be noted that, due to the conserved sequence homology between BCL-2 family members, this network of PPIs presents a significant challenge for selective protein labelling.

Tandem MS experiments revealed that the predominant labelling site of MCL-1 with minimal dimethylaniline label 6 was a single cysteine residue (Cys286), an amino acid not previously reported to react via this type of LDL chemistry.³⁶ Kodadek and co-workers reported that protein-protein crosslinking using Ru(II)(bpy)₃ reagents can be inhibited by the addition of excess cysteine (or tyrosine, tryptophan, methionine or histidine),³⁰ suggesting that thiols can trap or quench radicals formed upon photoexcitation. More importantly, Ru(II)(bpy)₃ and related complexes have been used for the formation of thiol radicals and their use in C-S bond formation, including on peptides. ³²-³⁴ In addition, the group of Finn demonstrated the use of Ru(II)(bpy)₃-mediated photolabelling of Tyr residues of viral capsid proteins with thiol derivatives,³⁵ suggesting that thiols can efficiently form C–S bonds with electron-rich aromatics in these types of reactions. It should be noted that the distance limits of SET-mediated protein labelling (and therefore residue selectivity) may depend on the contribution of different reaction pathways, which in turn may depend on the type of RTA used.³⁵

We found that the addition of APS as a co-oxidant was necessary for efficient protein labelling in vitro. However, previous literature describing intracellular protein labelling in the absence of APS proposes an alternative pathway whereby molecular oxygen acts as the electron acceptor and labelling is mediated by Ru(III) (which can be stabilised by consumption of superoxide, for example by addition of superoxide dismutase).³⁰,³² Therefore, future studies will focus on the development of Ru(II)(bpy)₃-
based LDL to study PPIs in cells and/or cell lysates, which may ultimately enable identification of novel transient/weak PPIs, without the need of protein overexpression.

Chemical labelling approaches to study PPIs complement enzyme-mediated proximity labelling approaches such as BioID and APEX. In comparison to traditional chemical crosslinking methods to study PPIs, photolabelling of proteins mediated by Ru(II)(bpy)$_3$-modified peptides such as 1 presents a number of potential advantages. Compared with non-specific reagents such as DSSO, SDA and Sulfo-SBED, ligand-directed labelling of a protein of interest within a complex mixture may facilitate analysis of its individual interactome. Additionally, reagent 1 is unreactive in biological media, activated only upon irradiation at a specific time point, whereas electrophilic groups such as NHS esters or maleimides are susceptible to hydrolysis/reaction with bulk nucleophiles in biological environments. The sub-stoichiometric quantities of Ru(II)(bpy)$_3$ peptide required for efficient labelling and short irradiation times (1 min) at longer wavelengths than UV-activated crosslinking reagents are also less likely to perturb the system under study. Based on reports by Kodadek et al and on our recent experience with bespoke LED-based irradiation systems for photoaffinity labelling, we expect that labelling with shorter irradiation times can be achieved in future studies. This would allow future dynamic interactome studies with high temporal resolution.

**Methods**

**Synthesis and characterisation.** Full synthetic procedures are available in the Supplementary Methods.

**Fluorescence anisotropy.** Assays were carried out in 384 well Optiplates and wells were read using a PerkinElmer EnVisionTM 2103 MultiLabel plate reader. Fluorescein-labelled peptides were examined using excitation and emission wavelengths of 480 nm and 535 nm, respectively (dichroic mirror 505 nm). All assays were performed in Tris buffer (50 mM Tris, 150 mM NaCl, 0.01% Triton X-100, pH 7.4). Direct titrations and competition assays were performed with minor modifications to those described previously and are detailed in full in the ESI.

**General procedure for photolabelling of recombinant MCL-1.** To a solution of MCL-1 (final concentration 5 μM) in ammonium acetate buffer (50 mM, pH 7.5) was added Ru(II)(bpy)$_3$-NOXA-B (final concentration 1 μM), RTA (final concentration 5-10 μM) and APS (final concentration 10 μM), and the mixture was incubated at r.t. for 5 min. The mixture was irradiated for 1 min at r.t., 5 cm from the light source (Kessil H150W-BLUE LED, 32W, 2 × lamps) and then immediately quenched by the addition of DTT (final concentration 10 mM) and analysed using ESI-MS and/or SDS-PAGE. Fluorescently modified peptides were analysed using a Molecular Imager ChemiDoc XRS System (Bio-Rad, CA). The details of specific labelling experiments are provided in the ESI.

**MS/MS identification of modified amino acid residue.** To a solution of MCL-1 (50 μM) in ammonium acetate buffer (50 mM, pH 7.5) was added Ru(II)(bpy)$_3$-NOXA-B 1 (final concentration 10 μM), RTA 6 (final concentration 10 μM) and APS (final concentration 10 μM) and the mixture was incubated at r.t. for 5 min. The mixture was irradiated for 1 min at r.t., 5 cm from light source (Kessil H150W-BLUE LED, 32W, 2 × lamps) and immediately quenched by the addition of DTT (final concentration 10 mM). The sample was split into two 50 μL aliquots. To each aliquot, a protease solution (Trypsin or Glu-C; Promega, Madison, WI); 20 ng μL$^{-1}$ in 25 mM ammonium bicarbonate) was added in a 1:50 ratio (protease:total protein content). Samples were incubated at 37 °C with shaking for 18 h. The digest reaction was stopped by adding 5 μL of 1% HCOOH, then subjected to purification using a Sep-pak 18 column. The Sep-pak column was equilibrated with 1 mL 0.1% TFA. 500 μL of 0.1% TFA was added to
the peptide digest, the mixture was passed through the column and the column was washed with 1 mL 0.1% TFA. Peptides were eluted from the column with 500 µL MeCN-H₂O 1:1 + 0.1% HCOOH. The eluant was dried by vacuum centrifugation and the peptides were reconstituted in 20 µL 0.1% TFA. LC separation of the peptide mixtures was performed on an ACQUITY M-Class UPLC (Waters UK, Manchester). 1 µL sample was loaded onto a Symmetry C18 trap column and washed with 1% MeCN/0.1% HCOOH for 5 min at 5 µL min⁻¹, then the peptides were separated on a HSS T3 C18 analytical column (Waters UK, Manchester) by gradient elution of 1-60% solvent B (0.1% HCOOH in MeCN) in A (0.1% HCOOH in H₂O) over 30 min at 0.3 µL min⁻¹. The peptides were analysed using a Xevo G2-XS Q-TOF mass spectrometer. Data processing was performed using the MassLynX v4.1 suite of software. Peptide MS/MS data from both trypsin and Glu-C digests were processed with PEAKS Studio (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) and searched against the amino acid sequence. 176.0951 Da was set as a variable modification on any residues to determine the position of the RTA 6 modification. MS mass tolerance was 10 ppm, and fragment ion mass tolerance was 0.05 Da. The false discovery rate was set to 1%.

Data availability

The authors declare that the data supporting the findings of this study are available within the article and Supplementary Information file, or from the corresponding author upon reasonable request.

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**Author contributions**

R.S.B. and A.J.W. conceived the project and supervised the research. H.A.B. carried out the synthesis of reagents, protein expression and purification, biophysical assays, protein labelling experiments and related data analysis. R.M.G carried out mass spectrometry-based peptide mapping to identify the MCL-1 labelling site. H.A.B., A.J.W. and R.S.B wrote the manuscript. All authors commented on the manuscript.
Additional Information

Supplementary Information Synthesis and characterisation of compounds, full experimental details, and additional data figures.
Proximity-induced photocatalytic labelling of MCL-1 by a BH3 ligand

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General comments

All chemical reagents were purchased from commercial suppliers and used without further purification. When used as reaction solvents, THF and CH$_2$Cl$_2$ were dried and deoxygenated using an Innovative Technology Inc. PureSolv® solvent purification system.

Flash column chromatography was carried out using silica (Merck Geduran silica gel, 35–70 μm particles) according to the method described by Still, Kahn and Mitra.\textsuperscript{1} Thin layer chromatography was carried out on commercially available pre-coated aluminium plates (Merck silica 2 8 8 0 Kieselgel 60 F$_{254}$). Analytical HPLC (small molecules) was performed on an Agilent 1290 Infinity Series equipped with a UV detector and a Hyperclone C$_{18}$ reverse phase column using MeCN/water (5→95%) containing 0.1% formic acid, at either 0.5 mL min$^{-1}$ over a period of five minutes or 1.0 mL min$^{-1}$ over a period of 30 minutes.

High resolution electrospray (ESI+) mass spectrometry was performed on a Bruker MaXis Impact QTOF mass spectrometer, and $m/z$ values are reported in Daltons to four decimal places. LC-ESI-MS data were recorded on an Agilent Technologies 1200 series HPLC combined with a Bruker HCT Ultra ion trap using 50 × 20 mm C$_{18}$ reverse phase columns using MeCN/water (5→95%) containing 0.1% formic acid. A flow rate of 1.5 mL min$^{-1}$ was used and $m/z$ values are given in Daltons to one decimal place.

\textsuperscript{1}H and $^{13}$C NMR spectra were recorded in deuterated solvents on a Bruker Avance 500 or Bruker Avance DPX 300. Chemical shifts are quoted in parts per million downfield of tetramethylsilane and referenced to residual solvent peaks (CDCl$_3$: $^1$H = 7.26 ppm, $^{13}$C = 77.16 ppm, DMSO-d$_6$: $^1$H = 2.50 ppm, $^{13}$C = 39.52 ppm) and coupling constants ($J$) are reported to the nearest 0.1 Hz. Assignment of spectra was based on expected chemical shifts and coupling constants, aided by COSY, HSQC and HMBC measurements where appropriate.

Fourier-transform infrared absorption spectroscopy (IR) was performed on Bruker Platinum-ATR system equipped with an Alpha FT-IR spectrometer. Maximum absorbances are reported for significant bands in cm$^{-1}$. 
Synthesis of Ru(II)(bpy)$_3$ complex for peptide capping

Scheme S1: Synthetic route to heteroleptic ruthenium(II) bipyridine complex 12 (prepared according to the previously reported method by Meyer et al.\textsuperscript{2})

Tris(2,2'-bpyridine)ruthenium(II) dichloride 10$^3$

To a solution of ruthenium(III) chloride hydrate (500 mg, 2.41 mmol) in DMF (7.5 mL) was added lithium chloride (717 mg, 16.90 mmol), followed by 2,2'-bipyridine (753 mg, 4.82 mmol) in three equal portions, and the reaction mixture was heated at reflux overnight. The reaction mixture was cooled to room temperature, acetone was added (40 mL) and the mixture was left at -4°C overnight. The brown microcrystals were filtered, washed with Et$_2$O (30 mL) and water (10 mL) to afford a brown solid (1.15 g, 98%). The crude product was used without further purification. HRMS (ESI): calcd. for C$_{20}$H$_{16}$Cl$_2$N$_4$NaRu [M+Na]$^+$ 506.9686, found 506.9685. Data consistent with literature.

Bis-(2,2'-bpyridine)-(4'-methyl-[2,2'-bpyridine]-4-carboxylic acid)ruthenium (II) bis-(hexafluorophosphate) 12$^2$

To a solution of crude 10 (905 mg, 1.87 mmol) in a mixture of EtOH (42 mL) and water (18 mL), was added 4-methyl-2,2'-bpyridine-4'-carboxylic acid (250 mg, 1.17 mmol), and the
reaction mixture was heated at reflux for 24 hours. The reaction mixture was cooled to room temperature, the solvents were removed in vacuo and the residue was re-suspended in water (30 mL) and filtered. The crude product was purified by FCC (SiO\(_2\), eluting with 100:20:1→100:20:1, v/v/v, MeCN:H\(_2\)O:KNO\(_3\)(sat.)), solvents were removed in vacuo, and the residue was re-dissolved in water (30 mL). Ammonium hexafluorophosphate (500 mg) was added in portions, resulting in the formation of a red-orange precipitate, which was collected via vacuum filtration and washed with H\(_2\)O (20 mL) to give the product as dark red crystals (740 mg, 43%). \(R_f\) 0.15 (100:20:1, v/v/v, MeCN:H\(_2\)O:KNO\(_3\)(sat.)); \(\delta_H\) (500 MHz, acetonitrile-d\(_3\)) 8.93 (1H, s, 3’-CH), 8.53 – 8.45 (5H, m, 3-CH, 3’’-CH), 8.05 (4H, m, 6-CH), 7.82 – 7.69 (6H, m, 4-CH, 6’-CH, 6’’-CH), 7.54 (1H, d, J 5.8, 5’-CH), 7.44 – 7.34 (4H, m, 5-CH), 7.24 (1H, d, J 5.5, 5’’-CH), 2.53 (3H, s, CH\(_3\)); \(\delta_C\) (125 MHz, acetonitrile-d\(_3\)) 167.6 (C(O)OH), 158.1 – 157.9 (C-2, C-2’, C-2’’), 152.8 (CH-6’), 152.7 (CH-4), 152.6 (C-4’), 151.65 (CH-5’), 146.8 (C-4’’), 138.7 (CH-6), 129.4 (CH-5’’), 128.6 (CH-5), 127.7 (CH-6’’), 126.3 (CH-3’), 125.2 (CH-3), 124.3 (CH-3’), 21.15 (CH\(_3\)); IR (neat, \(\nu_{max}/\text{cm}^{-1}\)) 3320, 1726, 1618, 1424, 1233, 825; HRMS (ESI): calcd. for C\(_{32}\)H\(_{26}\)N\(_6\)O\(_2\)Ru [M]\(^{2+}\) 315.0581, found 315.0583. Data consistent with literature.

**Synthesis of NOXA-B peptides**

**General comments**

All amino acids and resins were purchased from either Novabiochem (Merck) or Sigma-Aldrich. All amino acids were \(N\)-Fmoc protected and side chains protected with Boc (Lys); \(^{t}\)Bu (Asp, Glu, Ser, Thr, Tyr); Trt (Asn, Gln); Pbf (Arg). Synthesis of all peptides was performed using a microwave assisted automated peptide synthesiser (CEM, Liberty Blue). Peptide acetylation was performed manually. DMF used in peptide synthesis was of HPLC grade and from Sigma Aldrich. Peptides were synthesised on a 0.125 mmol scale. Lyophilisation was performed using a BenchTop Pro with Omnitronics™ (VirTis SP Scientific). Preparative HPLC was performed on an Agilent Technologies 1260 infinity controller in conjunction with a diode array detector. Analytical HPLC experiments were performed on an Agilent Technologies 1260 infinity controller in conjunction with a diode array detector. Mass spectrometry data were obtained on a Bruker Daltonics microTOF using electrospray ionisation (ESI) MS instruments as appropriate. FITC-NOXA-B (FITC-Ahx-AAQLRRIGDKVNLRRKLLN-NH\(_2\)), FITC-BID (FITC-Ahx-E\(^{80}\)DIIRNIARHLAQVGDNS\(_6\)DRSIW-NH\(_2\)) and Ac-BID (Ac-E\(^{80}\)DIIRNIARHLAQVGDNS\(_6\)DRSIW-NH\(_2\)) were prepared previously. 4,5
Scheme S2: Synthetic route to acetylated and Ru(II)(bpy)$_3$ derivatives of NOXA-B peptide. Cleavage cocktail "Reagent K" is composed of TFA:EDT:thioanisole:phenol:H$_2$O, 82:3:5:5:5.
Procedure for automated SPPS

Resin Loading: Clean reaction vessel; wash with DMF; transfer resin to reaction vessel; wash with DMF:CH₂Cl₂ (1:1); vessel draining.

Deprotection and Coupling: Add 20% piperidine in DMF (4 mL); microwave method (30 sec); wash with DMF (4 × 4 mL); drain; add amino acid (2.5 mL); add coupling reagent (1 mL); add base (0.5 mL); microwave method (1 min); wash through manifold to waste (2 mL); drain. For the deprotection and coupling of methods that did not use microwave assistance, the reactions were performed by agitation of the resin at r.t. for 10 min and 90 min, respectively. After the final residue, the resin was ejected from the reaction vessel and cleavage and deprotection was performed manually using methods A to D (section 5.3.2.3).

Methods for manual solid phase N-terminal chain elongation and capping

Method A: Deprotection of N-Fmoc protecting groups

N-terminal Fmoc protecting groups were removed by the addition of 20% piperidine: DMF (5 × 2 mL × 2 min), followed by rinsing the resin with DMF (5 × 2 mL × 2 min). Successful deprotection was determined by a positive colour test (Method B).

Method B: Kaiser Test

The Kaiser Test was used to determine successful coupling or deprotection of manually coupled residues. A few beads of resin were placed in a vial, two drops of each solution (1-3, see below) was added to the beads and the solution was heated to ca. 100 °C for 1 minute. Successful coupling was indicated by no change in colour of the beads, whereas successful deprotection was indicated by bright blue beads.

1) Ninhydrin (5% w/v) in ethanol
2) Phenol (80% w/v) in ethanol
3) 1 mM KCN (aq.) in pyridine (2% v/v)

Method C: N-terminal acetylation

Acetic anhydride (10 equiv.) and DIPEA (10 equiv.) were dissolved in DMF (1 mL) and the solution was transferred to the resin. After 2 h, the resin was drained, washed with DMF (3 × 2 mL × 2 min) and successful capping determined by a negative colour test (Method B).

Method D: Cleavage and deprotection of Rink amide MBHA resin
After elongation and N-terminal capping was complete, the resin was washed with DMF $(5 \times 2 \text{ mL} \times 2 \text{ min})$, CH$_2$Cl$_2$ $(5 \times 2 \text{ mL} \times 2 \text{ min})$, Et$_2$O $(5 \times 2 \text{ mL} \times 2 \text{ min})$ and dried under vacuum for 1 h. Peptides were simultaneously cleaved and side-chain deprotected using 'Reagent K' TFA:EDT:thioanisole:phenol:H$_2$O, 82:3:5:5:5 (1 \times 2 \text{ mL} \times 2 \text{ h}). The solution was precipitated in ice-cold Et$_2$O $(50 \text{ mL})$, placed in a centrifuge ($3000 \text{ rpm} \times 1 \text{ min}$) and the supernatant was removed. The precipitate was washed with ice-cold Et$_2$O $(3 \times 30 \text{ mL})$ and the washed precipitate was dried under a stream of nitrogen (1 h), before being dissolved in H$_2$O and lyophilised.

**Peptide purification**

Peptides were purified by preparative mass-directed HPLC using a Jupiter Proteo C$_{18}$ preparative column on an increasing gradient of acetonitrile in water + 0.1% HCOOH (v/v) at a flow rate of 10 mL min$^{-1}$. Crude peptides were suspended in acetonitrile at an approximate concentration of 20 mg mL$^{-1}$. Purification runs injected a maximum of 0.4 mL of crude peptide solution and were allowed to run for 30 min, with acetonitrile increasing at a stated gradient. The mass directed chromatography software Masshunter by ChemStation (Agilent) was used to allow the collection of the desired peptide by mass, with the eluent split into an Agilent 6120 Quadropole LC-MS which triggers collection of eluent at a programmed m/z. Fractions containing purified peptide were combined, concentrated in vacuo and lyophilised.

**Synthesis of Ru(II)(bpy)$_3$-NOXA-B peptide 1**

Ru(II)(bpy)$_3$-NOXA-B was synthesised on a 0.125 mmol scale on rink amide MBHA LL resin (0.35 mmol g$^{-1}$) using procedures described in section 5.3.2.2, all residues following isoleucine were double coupled. The N-terminus was elongated by dissolving Fmoc-6-aminohexanoic acid (5 equiv.), HCTU (5 equiv.) and DIPEA (5 equiv.) in DMF (2 mL) and the mixture was added to the resin, followed by agitation for 2 h. After removal of the reagents by filtration, the resin was washed with DMF $(3 \times 2 \text{ mL} \times 2 \text{ min})$ and the success of coupling determined by a negative colour test (Method B). Deprotection of the Fmoc-protected N-terminus then followed (Method A). The N-terminus was elongated by dissolving Ru(II)(bpy)$_3$PF$_6$ 12 (1.5 equiv.), PyBOP (3 equiv.) and DIPEA (5 equiv.) in DMF (2 mL) and the mixture was added to the resin, followed by agitation for 2 h (double coupling). After removal of the reagents by filtration, the
resin was washed with DMF (3 × 2 mL × 2 min) and the success of coupling determined by a negative colour test (Method B). The peptide was simultaneously cleaved and side-chain deprotected using Method D. Ru(II)(bpy)₃-NOXA-B 1 was purified using mass-directed HPLC over a gradient of 15-25% acetonitrile in H₂O + 0.1% HCOOH, to give (3.9 mg, 2.6%) of the final peptide.

**Figure S1:** Analytical HPLC data for Ru(II)(bpy)₃-NOXA-B peptide 1.

**Figure S2:** HRMS (ESI) spectrum for Ru(II)(bpy)₃-NOXA-B peptide 1.

**Table S1:** Tabulated HRMS data for Ru(II)(bpy)₃-NOXA-B peptide 1 showing the expected (Exp) and observed (Obs) masses for the multiple charge states.

| Peptide          | [M+3H]³⁺ Obs  | [M+3H]³⁺ Exp  | [M+4H]⁴⁺ Obs  | [M+4H]⁴⁺ Exp |
|------------------|---------------|---------------|---------------|---------------|
| Ru(II)bpy)₃-NOXA-B | 976.5187      | 976.5162      | 732.6371      | 732.6391      |
Synthesis of Ac-NOXA-B 4

Ac-NOXA-B 4 was synthesised on a 0.125 mmol scale on rink amide MBHA LL resin (0.35 mmol g\(^{-1}\)) using procedures described in section 5.3.2.2, all residues following isoleucine were double coupled. The N-terminus was acetylated as described in section 5.3.2.3 (Method C) and the peptide was simultaneously cleaved and side-chain deprotected using Method D. Ac-NOXA-B 4 was purified using mass-directed HPLC over a gradient of 5-50% acetonitrile in H\(_2\)O + 0.1% HCOOH, to give (4.1 mg, 4.8%) of the final peptide.

**Figure S3:** Analytical HPLC data for Ac-NOXA-B peptide 4.

**Figure S4:** HRMS (ESI) spectrum for Ac-NOXA-B peptide 4.
Table S2: Tabulated HRMS data for Ac-NOXA-B peptide 4 showing the expected (Expd) and observed (Obsd) masses for the multiple charge states.

| Peptide     | [M+3H]\(^{3+}\) Obsd | [M+3H]\(^{3+}\) Expd | [M+4H]\(^{4+}\) Obsd | [M+4H]\(^{4+}\) Expd |
|-------------|-----------------------|-----------------------|-----------------------|-----------------------|
| wt NOXA-B   | 750.1231              | 749.7889              | 562.8446              | 562.2762              |

Synthesis of Radical Trapping Agents (RTAs)

Scheme S3: Synthetic routes to radical trapping agents 2 and 3. Both fluorescent (2, TAMRA-RTA) and biotinylated (3, biotin-RTA) labels were synthesised from common building block 18, in a divergent manner.

Scheme S4: Synthesis of minimal RTA 6, via acetylation of N,N-dimethyl-p-phenylenediamine 16.
**Tert-butyl(6-((4-(dimethylamino)phenyl)amino)-6-oxohexyl)carbamate 17**

To a solution of Boc-Ahx-OH (500 mg, 2.16 mmol) in THF (8 mL) at 0 °C (ice-bath) was added isobutyl chloroformate (305 µL, 2.36 mmol) slowly, then N-methylmorpholine (402 µL, 3.65 mmol) slowly. The resulting solution was stirred for 30 min at 0 °C, then allowed to warm to ambient temperature. After 1 h, a solution of N,N-dimethyl-p-phenylenediamine (478 mg, 3.51 mmol) in THF (0.5 mL) was added (solution turned dark green/brown) and the reaction mixture was stirred at ambient temperature overnight. The reaction mixture was quenched with 1 N HCl (ca. 5 mL) and washed with EtOAc (3 × 3 mL). A solution of 6 N NaOH was added to the mixture to become basic (pH 8). The product was extracted with EtOAc (5 × 10 mL), the organic layers were dried (Na₂SO₄), filtered and concentrated in vacuo. The crude product was purified by FCC (SiO₂, Biotage, 40 g, eluting with 12-70% EtOAc-Hex) to give the **title compound** as a purple solid (558 mg, 74%). Rᵣ 0.31 (1:1 EtOAc-Hex); δ_H (400 MHz, CDCl₃) 7.43 (1H, s, NH), 7.38 – 7.32 (2H, app. d, J 9.0, 2'-CH₂), 6.74 – 6.62 (2H, app. d, J 9.0, 3'-CH₂), 4.70 – 4.55 (1H, s, NH), 3.09 (2H, m, 6-CH₂), 2.89 (6H, s, N-CH₃), 2.30 (2H, t, J 7.5, 2-CH₂), 1.71 (2H, p, J 7.5, 3-CH₂), 1.54 – 1.30 (13H, m, O'Bu-CH₂, 4-CH₂, 5-CH₂); δ_C (100 MHz, CDCl₃) 171.1 (C-1), 156.2 (C(O)-tBu), 148.0 (C-4'), 128.2 (C-1'), 121.9 (CH-2'), 113.3 (CH-3'), 79.2 (C-1Bu), 41.1 (CH₃-N), 40.5 (CH₂-6), 37.4 (CH₂-2), 29.9 (CH₂-3), 28.6 (CH₃-tBu), 126.5 (CH₂-5), 25.4 (CH-4);

IR (neat, ν_max/cm⁻¹) 3373, 2932, 1649, 1513, 1159; HRMS (ESI): calcd. for C₁₉H₃₅N₃NaO₃ [M+Na]^+ 372.2258, found 372.2257.
6-amino-\(\text{N-}(4\text{-}(\text{dimethylamino})\text{phenyl})\text{hexanamide} \text{18}

\[
\begin{align*}
&\begin{array}{cccccccc}
1 & 2 & 3 & 4 & 5 & 6 & NH_2
\end{array} \\
&\begin{array}{cccccccc}
& & & & & & \\
3' & 4' & & & & & \\
& & & & & & \\
2' & 6 & & & & & \\
& & & & & & \\
1' & & & & & & \\
\end{array}
\end{align*}
\]

To a solution of \text{17} (1 g, 2.87 mmol) in \text{CH}_2\text{Cl}_2 (10 mL) was added trifluoroacetic acid (10 mL) and the reaction mixture was stirred at ambient temperature for 1.5 hours. The solvents were removed \textit{in vacuo} to give a purple oil. The crude product was purified by FCC (SiO\textsubscript{2}, eluting with \text{CH}_2\text{Cl}_2-\text{MeOH-NEt}_3 \xrightarrow{95:4:1\rightarrow90:9:1} \text{to afford the title compound as a pale purple waxy solid (681 mg, 95%).} \]

\[R_f \text{0.08 (CH}_2\text{Cl}_2-\text{MeOH-NEt}_3 \xrightarrow{90:9:1}\}; \delta_H (400 MHz, \text{CDCl}_3) 7.70 \text{ (1H, s, NH), 7.41 – 7.30 (2H, m, 2'-CH)}, 6.74 – 6.62 (2H, app. d, J 9.0, 3'-CH), 2.88 (6H, m, N-CH\text{3}), 2.68 (2H, t, J 6.9, 6-CH\text{2}), 2.30 (4H, m, 2-CH\text{2}, NH\text{2}), 1.70 (2H, p, J 7.5, 3-CH\text{2}), 1.55 – 1.30 (4H, m, 4-CH\text{2}, 5-CH\text{2}); \delta_C (100 MHz, \text{CDCl}_3) 171.4 \text{ (C-1), 148.0 (C-4'), 128.2 (C-1'), 121.9 (CH-2'), 113.2 (CH-3'), 41.8, (CH-2-6), 41.1 (CH\text{3}-N), 37.4 (CH\text{2}-2), 32.9 (CH\text{2}-5), 26.5 (CH\text{2}-4), 25.6 (CH\text{2}-3); IR (neat, \nu_{max}/cm^{-1}) 3282, 2930, 1644, 1517, 1315; HRMS (ESI): calcd. for C\text{14}H\text{24}N\text{3}O \text{[M+H]+}} 250.1914, \text{found 250.1912.}

\[2-(6\text{-}(\text{dimethylamino})\text{-3-(dimethyliminio)-3H-xanthen-9-yl)-5-((6-\text{(4-dimethylamino) phenyl)amino)-6-oxohexyl)carbamoyl}benzoate (TAMRA-RTA) 2

\[
\begin{align*}
&\begin{array}{cccccccc}
10 & 11 & 12 & 13 & 9a & 8a & 10a & 4a
\end{array} \\
&\begin{array}{cccccccc}
& & & & & & & \\
7 & 6 & & & & & & \\
& & & & & & & \\
& & & & & & & \\
& & & & & & & \\
& & & & & & & \\
\end{array}
\end{align*}
\]

To a solution of amine \text{18} (50 mg, 0.20 mmol) in DMF (1 mL) was added TAMRA-NHS (138 mg, 0.26 mmol) and NE\text{3} (56 \muL, 0.40 mmol) and the mixture was stirred at ambient temperature for 20 hours. The solvent was removed \textit{in vacuo} and the residue was re-dissolved in CHCl\text{3} (5 mL). The organic layer was washed with water (1 \times 2 mL), saturated aqueous NaHCO\text{3} (aq., 3 \times 2 mL), brine (1 \times 2 mL), dried over anhydrous Na\text{2}SO\text{4}, filtered and concentrated to give a purple solid. The crude product was purified by FCC (SiO\textsubscript{2}, eluting with CH\text{2}Cl\text{2}-MeOH 19:1→9:1) to afford the title compound as a purple solid (11 mg, 12%). \[R_f \text{0.12 (CH}_2\text{Cl}_2-\text{MeOH 9:1}; \delta_H (500 MHz, \text{CDCl}_3) 8.43 \text{ (1H, s, 15-CH}, 8.08 \text{ (1H, d, J 8.2, 11-CH}, 7.37 – 7.30 \text{ (2H, m, 2'-CH)}, 7.14 \text{ (1H, d, J 7.9, 12-CH)}, 6.90 \text{ (2H, d, J 8.9, 1'''-CH, 8'''-CH)}, 6.66 – 6.60 \text{ (2H, m, 3'-CH)}, 6.61 – 6.46 \text{ (5H, m, 2''-CH, 4''-CH, 5''-CH, 7''-CH, NH}), 3.42 \text{ (2H, t, J 6.8, 6-CH\text{2}), 3.02 (6H, s, rhodamine-N-CH\text{3}), 2.89 (6H, s, rhodamine-N CH\text{3}), 2.82 (6H, s, N-CH\text{3}), 2.28 (2H, t, J 7.5, 2-CH\text{2}), 1.68 (4H, m, 3-CH\text{2}, 5-CH\text{2}), 1.44 (2H, p, J 7.7, 4-CH\text{2}); IR}
(neat, \(v_{\text{max}}/\text{cm}^{-1}\)) 3496, 2998, 2853, 1651, 1493, 1320, 1058; HRMS (ESI): calcd. for \(\text{C}_{39}\text{H}_{45}\text{N}_{5}\text{O}_{5}\)[M+2H]^{2+} 331.6705, found 331.6705.

\(N\)-(4-(dimethylamino)phenyl)-6-(5-((4S)-2-oxohexahydro-1H-thienol[3,4-d]imidazol-4-yl)pentanamido)hexanamide (biotin-RTA) 3

To a solution of D-biotin (421 mg, 1.72 mmol) in CHCl\(_3\) (20 mL) was added EDCI (331 mg, 1.72 mmol), HOBT (233 mg, 1.72 mmol), amine 18 (286 mg, 1.15 mmol) and DIPEA (300 \(\mu\)L, 1.72 mmol), and the reaction mixture was stirred at ambient temperature overnight. The solvents were removed \(\text{in vacuo}\) to give a pale purple solid. The crude product was purified by FCC (SiO\(_2\), eluting with CHCl\(_3\)-MeOH 19:1→9:1) to afford the title compound as a colourless solid (182 mg, 33%).

\(R_f\) 0.32 (CHCl\(_3\)-MeOH 9:1); \(\delta_H\) (400 MHz, CDCl\(_3\)) 7.56–7.39 (2H, app. d, \(J_9.0, 2'-\text{CH}\)), 6.85 (2H, app. d, \(J_8.9, 3'-\text{CH}\)), 4.59 (1H, dd, \(J_7.9, 4.8, \text{biotin-CH}\)), 4.39 (1H, dd, \(J_7.9, 4.4, \text{biotin-CH}\)), 3.48 (1H, s, \(\text{biotin-CH}\)), 3.28 (3H, m, 6-\(\text{CH}_2\), \(\text{biotin-CH}\)), 3.16–2.86 (6H, m, N-\(\text{CH}_3\)), 2.82 (1H, d, \(J_{12.8}, \text{biotin-CH}\)), 2.45 (2H, t, \(J_{7.4, 2-\text{CH}}\)), 2.29 (2H, t, \(J_{7.1, 8-\text{CH}}\)), 1.90–1.59 (8H, m, 3-\(\text{CH}_2\), 9-\(\text{CH}_2\), 10-\(\text{CH}_2\), 11-\(\text{CH}_2\)), 1.48–1.34 (4H, m, 4-\(\text{CH}_2\), 5-\(\text{CH}_2\)); \(\delta_C\) (100 MHz, CDCl\(_3\)) 175.3 (C-1), 173.4 (C-7), 165.2 (C(O)-biotin), 148.7 (C-4'), 129.4 (C-1'), 122.5 (CH-2'), 114.2 (CH-3'), 62.7(CH-biotin), 60.9 (CH-biotin), 56.4 (CH-biotin), 41.1 (CH-\(\text{N}\)), 40.8 (CH\(_2\)-biotin), 39.7 (CH\(_2\)-6), 37.2 (CH\(_2\)-2), 36.4 (CH\(_2\)-11), 29.5–28.8 (CH\(_2\)-3, CH\(_2\)-9, CH\(_2\)-10), 27.0 (CH\(_2\)-11), 26.3 (CH\(_2\)-4 or CH\(_2\)-5), 26.0 (CH\(_2\)-4 or CH\(_2\)-5); IR (neat, \(v_{\text{max}}/\text{cm}^{-1}\)) 3283, 2927, 1697, 1645, 1518, 1458, 1260; HRMS (ESI): calcd. for \(\text{C}_{24}\text{H}_{38}\text{N}_{5}\text{O}_{3}\text{S}[\text{M+H}]^+\) 476.2690, found 476.2688.

\(N\)-(4-(Dimethylamino)phenyl)acetamide (minimal-RTA) 6

To a solution of \(N,N\)-dimethyl-p-phenylenediamine (100 mg, 0.73 mmol) in CH\(_2\)Cl\(_2\) (0.5 mL) was added triethylamine (50 \(\mu\)L, 0.36 mmol) and \(N,N\)-dimethyl-4-aminopyridine (2 mg, 0.02 mmol), and the reaction mixture was stirred for 5 min at ambient temperature. Acetic anhydride (140 \(\mu\)L, 1.50 mmol) was added dropwise to the reaction mixture, and the resulting mixture was stirred for 2 hours at ambient temperature. The reaction was quenched with water (20 mL) and the product was extracted with CH\(_2\)Cl\(_2\) (3 \(\times\) 5 mL). The combined organic layers were washed with water (1 \(\times\) 5 mL), dried over anhydrous Na\(_2\)SO\(_4\), filtered and concentrated
in vacuo to give a brown solid. The crude product was purified by FCC (SiO$_2$, eluting with 50:50→100:0 EtOAc-Hex) to afford the title compound as a grey powder (89 mg, 68%). $R_f$ 0.15 (EtOAc-Hexane 1:1); $\delta_H$ (400 MHz, DMSO-$d_6$) 9.58 (1H, s, NH), 7.42 – 7.31 (2H, app. d, $J$ 9.1, 2-CH$_2$), 6.73 – 6.60 (2H, app. d, $J$ 9.0, 3-CH$_2$), 2.83 (6H, s, N-CH$_3$), 1.97 (3H, s, CH$_3$); $\delta_C$ (100 MHz, DMSO-$d_6$) 167.3 (C=O), 146.9 (C-4), 129.3 (C-1), 120.5 (CH$_2$-2), 112.7 (CH$_2$-3), 40.5 (CH$_3$-N), 23.7 (CH$_3$); IR (neat, $\nu_{max}$/cm$^{-1}$) 3183, 2803, 1641, 1519, 1320; HRMS (ESI): calcd. for C$_{20}$H$_{28}$N$_4$NaO$_2$ [2M+Na]$^+$ 379.2106, found 379.2104. Data consistent with literature.
Expression and purification of MCL-1, BCL-xL and hDM2

Expression and purification of MCL-1, BCL-xL and hDM2 protein was performed as previously described.⁵,⁷,⁸
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A resolving gel of appropriate percentage was prepared according to *Error! Reference source not found.* 12% gels were used in analysis of MCL-1 expression and purification, 15% gels were used in the analysis of all photolabelling reactions. Following addition of tetramethylethylenediamine (TEMED), the solution was thoroughly mixed and immediately added to the BioRad tetragel apparatus. A layer of propan-2-ol (1 mL) was applied to the top of the gel. When the resolving gel had set, the propan-2-ol was removed and a stacking gel was prepared according to *Table S3*. Once the TEMED was added the solution was thoroughly mixed, immediately added to the top of the resolving gel and comb with a suitable number of lanes was inserted into the stacking gel.

*Table S3: SDS-PAGE gel recipes*

| Components          | Resolving gel 12% | Resolving gel 15% | Stacking gel 15% |
|---------------------|------------------|------------------|-----------------|
| 1.5 M Tris (pH 8.8) | 2.53 mL          | 2.53 mL          | -               |
| 0.5 M Tris (pH 6.8) | -                | -                | 0.95 mL         |
| 40 % (w/v) acrylamide | 3.00 mL         | 3.75 mL         | 0.62 mL         |
| 10% SDS             | 100 µL           | 100 µL           | 50 µL           |
| H₂O                 | 4.25 mL          | 3.50 mL         | 3.30 mL         |
| 10% APS             | 100 µL           | 100 µL          | 50 µL           |
| TEMED               | 10 µL            | 10 µL           | 10 µL           |

Once set, the gel was placed inside an electrophoresis tank and SDS running buffer was added. Samples were mixed with an equal volume of loading buffer, heated to 95 °C for 10 minutes and loaded onto the gel. Electrophoresis was performed at a constant voltage of 180V for approximately 50 minutes, or until the loading buffer had reached the bottom of the gel. Proteins were visualised using Coomassie Blue stain (45% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (w/v) Coomassie Brilliant Blue R-250) and destained (30% (v/v) methanol, 10% (v/v) acetic acid), or alternatively using Instant Blue (TripleRed).
Fluorescence anisotropy assays

General remarks

Fluorescence anisotropy assays were run in 384 well Optiplates and scanned using a Perkin Elmer EnVision™ 2103 MultiLabel plate reader. Fluorescein labelled peptides used an excitation and emission wavelength of 480 nm (30 nm bandwidth) and 535 nm (40 nm bandwidth) respectively. All assays were run in Tris buffer (50 mM Tris, 150 mM NaCl, 0.01% Triton X-100, pH 7.4) with additives where described.

Direct binding assays

Processing of fluorescence anisotropy data

The data obtained for both the $P$ (perpendicular intensity) and $S$ (parallel intensity) channels were corrected by subtracting the corresponding control wells, and the resulting values were used to calculate intensity (Eq. 1) and anisotropy (Eq. 2) for each well (using Microsoft Excel). These data were transferred into OriginPro 8.5, where a plot of anisotropy against protein concentration was fitted using a logistic model (Eq. 3) to obtain the minimum ($r_{\text{min}}$) and maximum ($r_{\text{max}}$) values of anisotropy. These values were used to determine the fraction of labelled peptide bound to the protein (fraction ligand bound, $L_b$, Eq. 4), and fitted (Eq. 5) in OriginPro 8.5 to determine the dissociation constant, $K_d$.

$$I = 2PG + S \quad \text{Equation 1}$$

Where $I$ is the total intensity, $G$ is an instrument factor which was set to 1, and $r$ is the anisotropy.

$$r = \frac{S - PG}{I} \quad \text{Equation 2}$$

$$y = r_{\text{max}} + \frac{r_{\text{min}} - r_{\text{max}}}{1 + (x/x_0)^p} \quad \text{Equation 3}$$

$$Lb = \frac{r - r_{\text{min}}}{(\lambda(r_{\text{max}} - r) + r - r_{\text{min}})} \quad \text{Equation 4}$$

Where $x_0$ is the midpoint, $p$ is the power and $\lambda$ is the ratio of $I_{\text{bound}}/I_{\text{unbound}}$ and is equal to 1.

$$y = \frac{(K_d + x + [FL]) - \sqrt{((K_d + x + [FL])^2 - 4x[FL])}}{2} \quad \text{Equation 5}$$

Where $Lb$ is the fraction ligand bound, $[FL]$ is the concentration of fluorescent ligand, $y$ is $Lb^[FL]$, $x = [\text{added titrant}]$. 

17
MCL-1/FITC-NOXA-B direct titration

Titration of MCL-1 into NOXA-B was performed in a 384 well plate in Tris Buffer (50 mM Tris, 150 mM NaCl, 0.01% Triton X-100, pH 7.4) with the concentration of MCL-1 starting at 10 µM, diluted over 24 points in a 1/2 regime with [FITC-NOXA-B] fixed at 25 nM. Plates were read after 1 hour and 20 hours incubation. Assays were run in triplicate (both test wells and control wells).

Figure S6: Direct titration of MCL-1 into FITC-NOXA-B to give a $K_d$ of 11 ± 3 nM (error stated is SEM) that corresponds to that described previously (13 ± 2 nM). Error bars represent the standard deviation of three replicates.

BCL-Xₐ/FITC-BID direct titration

Titration of BCL-Xₐ into FITC-BID was performed in a 384 well plate in Tris Buffer (50 mM, Tris, 150 mM NaCl, 0.01% Triton-X-100, pH 7.4) + 0.02 mg/mL BSA with the concentration of BCL-Xₐ starting from 10 µM, diluted over 24 points in a 1/2 regime with [FITC-BID] fixed at 25 nM. Plates were read after 1 hour incubation and 20 hours incubation, data shown is from 1 hour incubation (assay died at 20 hour time point). Assays were run in triplicate (both test wells and control wells).
Figure S7: Direct titration of BCL-X$_L$ into a constant concentration of FITC-BID to give a $K_d$ of $54 \pm 8$ nM (error stated is SEM) that corresponds to that described previously ($79 \pm 6$ nM).\textsuperscript{5} Error bars represent the standard deviation of three replicates.

Competition assays

General remarks

All competition assays were performed in 384 well plates with the concentration of peptide competitor serially diluted over 16 or 24 points in a 1/2 regime with [tracer] fixed at 25 nM. The assays consisted of three test rows (containing protein, tracer and competitor), and three control rows (tracer peptide was replaced with buffer). The intensity, $I$, and anisotropy, $r$, were calculated using equations 1 and 2, respectively. A plot of anisotropy against competitor concentration was plotted in OriginPro 8.5 and fitted using Eq. 4 to determine an IC$_{50}$ value.
Competition of MCL-1/FITC-NOXA-B interaction by Ru(II)(bpy)$_3$NOXA-B and Ac-NOXA-B

MCL-1/FITC-NOXA-B competition assays were performed in Tris Buffer (50 mM, Tris, 150 mM NaCl, pH 7.4) + 0.01% Triton-X-100 with [MCL-1] fixed at 75 nM. Assays were run in triplicate (both test wells and control wells). Plates were read after 1 h and 20 h incubation.

Competition of BCL-X$_L$/FITC-BID interaction by Ru(II)(bpy)$_3$NOXA-B

BCL-X$_L$/FITC-BID competition assays were performed in Tris Buffer (50 mM, Tris, 150 mM NaCl, 0.01% Triton-X-100, pH 7.4) + 0.02 mg/mL BSA with [BCL-X$_L$] fixed at 50 nM. Both control wells and test wells were run in triplicate. Plates were read after 1 h and 20 h incubation, data shown is from 20 h incubation.
Ligand-directed photolabelling experiments

Labelling of recombinant MCL-1 with TAMRA-RTA
To a solution of MCL-1 (final concentration 5 μM) in ammonium acetate buffer (50 mM, pH 7.5) was added Ru(II)(bpy)$_3$-NOXA-B (final concentration 1 μM), TAMRA-RTA (final concentration 5 μM) and APS (final concentration 10 μM), and the mixture was incubated at r.t. for 5 min. For the competition experiment, Ac-BID (final concentration 0-1000 μM) was added and the mixture was incubated for 10 min prior to incubation with Ru(II)(bpy)$_3$-NOXA-B. The mixture was irradiated for 1 min at r.t., 5 cm from the light source (Kessil H150W-BLUE LED, 32W, 2 × lamps). The reaction was immediately quenched by the addition of DTT (final concentration 10 mM) and analysed using ESI-MS and/or SDS-PAGE. Fluorescently modified peptides were analysed using a Molecular Imager ChemiDoc XRS System (Bio-Rad, CA). After obtaining the fluorescence image, the same gel was stained with Coomassie Brilliant Blue (CBB) and visualised on a Molecular Imager ChemiDoc XRS System. ImageJ was used to quantify bands on the gel.

![Image of photolabelling set-up](image.png)

**Figure S8:** Photolabelling set-up: two Kessil H150W-BLUE LED lamps facing each other, parallel to base of the box containing the samples in Eppendorf tubes.
Figure S9: Photolabelling of MCL-1 with TAMRA-RTA 2. Fluorescence image (top) and Coomassie Brilliant Blue (CBB, bottom) stained SDS-PAGE gels; fluorescence image shows MCL-1 only labelled when 1 min $h\nu$, 5 µM TAMRA-RTA and 1 µM Ru(II)(bpy)$_3$-NOXA-B are present (5 µM MCL-1 and 10 µM APS used), CBB stain shows MCL-1 for all conditions.

| Lane | 1 | 2 | 3 | 4 | 5 |
|------|---|---|---|---|---|
| 5 µM TAMRA-RTA 2 | + | - | + | + | + |
| 1 µM Ru(II)(bpy)$_3$-NOXA-B | + | + | - | + | + |
| 10 µM APS | + | + | + | - | + |
| $hv$ (min) | 1 | 1 | 1 | 0 | 0 |

Lane 1: MCL-1, TAMRA-RTA, 20 kDa, 15 kDa
Lane 2: MCL-1, TAMRA-RTA, 20 kDa, 15 kDa
Lane 3: MCL-1, TAMRA-RTA, 20 kDa, 15 kDa
Lane 4: MCL-1, TAMRA-RTA, 20 kDa, 15 kDa
Lane 5: MCL-1, TAMRA-RTA, 20 kDa, 15 kDa
Selective labelling of MCL-1 with TAMRA RTA over BCL-X<sub>L</sub>

|                | BCL-X<sub>L</sub> | MCL-1 |
|----------------|-------------------|-------|
| 5 µM TAMRA label 2 | + - + + +       | + - + + + |
| 1 µM Ru(II)(bpy)<sub>3</sub>-NOXA-B | + - - + +       | + + - + + |
| 10 µM APS        | + + + - +       | + + + - + |
| <i>hv</i> (min)  | 1 1 1 1 0       | 1 1 1 1 0 |
| Lane             | 1 2 3 4 5       | 6 7 8 9 10 |

**Figure S10:** Photolabelling of BCL-X<sub>L</sub> and MCL-1 with TAMRA RTA. Fluorescence image (top) and Coomassie Brilliant Blue (CBB, bottom) stained SDS-PAGE gels; fluorescence image shows MCL-1 only labelled when 1 min <i>hv</i>, 5 µM TAMRA RTA and 1 µM Ru(II)(bpy)<sub>3</sub>-NOXA-B are present (5 µM MCL-1 and 10 µM APS used), CBB stain shows BCL-X<sub>L</sub> and MCL-1 for all conditions.
**Figure S11**: MCI-1 is selectively labelled with TAMRA-RTA 3 over BCL-X<sub>L</sub> in a 1:1 mixture of proteins, while BCL-X<sub>L</sub> is not labelled in solution on its own. Conditions: 5 µM protein, 5 µM TAMRA-RTA and 1 µM Ru(II)(bpy)<sub>3</sub>-NOXA-B, 10 µM APS, 1 min hv.
Figure S12: MCl-1 is selectively labelled with TAMRA-RTA 3 over BCL-X<sub>L</sub> in a 1:1 mixture of proteins, while BCL-X<sub>L</sub> is not labelled in solution on its own. Conditions: 5 µM protein, 5 µM TAMRA-RTA and 1 µM Ru(II)(bpy)<sub>3</sub>-NOXA-B, 10 µM APS, 1 min hv.
**Figure S13:** Both MCL-1 and BCL-X<sub>i</sub> are labelled in a 1:1 mixture of proteins (lanes 3, 4 and 5) when high concentrations of TAMRA-RTA 2 are used. Conditions: 5 µM protein, 1 µM Ru(II)(bpy)<sub>3</sub>-NOXA-B, 500 µM TAMRA-RTA, 10 µM APS, 50 mM (NH₄)HCO₃ (pH 7.4), 1 min hv.
Selective labelling of MCL-1 with TAMRA-RTA over hDM2

**Figure S14:** MCL-1 is selectively labelled in a 1:1 mixture of MCL-1 and hDM2 (lane 3). Conditions: 5 µM protein, 1 µM Ru(II)(bpy)$_3$-NOXA-B, 5 µM TAMRA-RTA, 10 µM APS, 50 mM (NH$_4$)HCO$_3$ (pH 7.4), 1 min hv.
**Figure S15:** Competition experiment using Ac-NOXA-B. Conditions: 5 µM MCL-1, 5 µM TAMRA-RTA, 1 µM Ru(II)(bpy)$_3$-NOXA-B, 10 µM APS, 1 min hv, 0-100 µM Ac-NOXA-B.
Figure S16: Competition experiments with Ac-BID. Conditions: 5 μM MCL-1, 5 μM TAMRA-RTA, 1 μM Ru(II)(bpy)$_2$-NOXA-B, 10 μM APS, 1 min hν, 0-1000 μM Ac-NOXA-B.

Labelling of recombinant MCL-1 with biotin-RTA

To a solution of MCL-1 (5 μM) in ammonium acetate buffer (50 mM, pH 7.5) was added Ru(II)(bpy)$_2$-NOXA-B (final concentration 1 μM), Biotin-RTA (final concentration 5 μM) and APS (final concentration 10 μM) and the mixture was incubated at r.t. for 5 min. The mixture was irradiated for 1 min at r.t., 5 cm from light source (Kessil H150W-BLUE LED, 32W, 2 × lamps). The reaction was immediately quenched by the addition of DTT (final concentration 10 mM) and analysed using ESI-MS and/or SDS-PAGE.
**ESI-MS spectra of MCL-1 labelling reactions using biotin-RTA**

*Conditions:* 5 µM MCL-1, 1 µM Ru(II)(bpy)$_3$-NOXA-B 1, 5 µM biotin-RTA 3, 10 µM APS, 1 min *hv*.

*Conditions:* 5 µM MCL-1, 0 µM Ru(II)(bpy)$_3$-NOXA-B 1, 5 µM biotin-RTA 3, 10 µM APS, 1 min *hv*.

*Conditions:* 5 µM MCL-1, 1 µM Ru(II)(bpy)$_3$-NOXA-B 1, 5 µM biotin-RTA 3, 0 µM APS, 1 min *hv*.

*Conditions:* 5 µM MCL-1, 1 µM Ru(II)(bpy)$_3$-NOXA-B 1, 0 µM biotin-RTA 3, 10 µM APS, 1 min *hv*.

*Conditions:* 5 µM MCL-1, 1 µM Ru(II)(bpy)$_3$-NOXA-B 1, 5 µM biotin-RTA 3, 10 µM APS, 0 min *hv*.

**Figure S17:** Deconvoluted ESI-MS spectra from photolabelling reactions under various conditions. Notably, the reaction without Ru(II)(bpy)$_3$-NOXA-B 1 gives no biotin-labelled protein.
Pull-down of biotinylated MCL-1

The crude labelling mixture was incubated with avadin-agarose beads (50 µL) at r.t. for 2 hours with gentle agitation (input lanes 1-4 and their respective pull-down lanes 6-9). Note that for input lane 5/pull-down lane 10, excess biotin-RTA 3 was removed from the crude labelling mixture using a protein concentrator (10 kDa MWCO) prior to incubation with avidin-agarose beads. Following removal of the supernatant, the beads were washed twice with PBS (100 µL), then boiled at 90 °C for 10 mins in sample loading buffer to elute bound proteins. Samples were separated by SDS-PAGE: the gel (15% acrylamide) was run at a constant voltage of 180 V for 45 min, then the gel was stained with Coomassie Brilliant Blue (CBB) and the image obtained on a Molecular Imager ChemiDoc XRS.

![Figure S18: Isolation of biotinylated MCL-1 from crude labelling mixture through pull-down with avadin-agarose beads (CBB stained gel). Conditions: 5 µM MCL-1, 0-100 µM biotin-RTA 3, 1 µM Ru(II)(bpy)$_3$-NOXA-B, 10 µM APS, 1 min irradiation, 50 mM (NH$_4$)$_2$HCO$_3$ (pH 7.4). Biotinylated MCL-1 was pulled-down when 20 µM and 10 µM biotin-RTA 3 was used (lanes 7 and 8, respectively), in addition to when 100 µM biotin-RTA 3 was used and the excess label 3 was removed using a protein concentrator (lane 10). 50 µL avidin-agarose beads was used in pull-down experiments.](image-url)
ESI-MS analysis of MCL-1 photolabelling reactions

**Figure S19:** ESI-MS data showing photolabelling of MCL-1 with TAMRA-RTA after 1 minute of irradiation. Conditions: 5 µM protein, 1 µM Ru(II)(bpy)$_3$-NOXA-B, 5 µM TAMRA-RTA, 10 µM APS, 50 mM (NH$_4$)HCO$_3$ (pH 7.4). Masses indicative of oxidation of amino acid residues on unlabelled MCL-1 are present in the mass spectrum (mass increments of +16 Da).

**Figure S20:** a) ESI-MS data showing photolabelling of MCL-1 with biotin-RTA after 15 mins irradiation. Conditions: 5 µM protein, 1 µM Ru(II)(bpy)$_3$-NOXA-B, 5 µM TAMRA-RTA, 10 µM APS, 50 mM (NH$_4$)HCO$_3$ (pH 7.4). b) Zoom in on MCL-1 peak, showing mass increments of +16 Da, indicating oxidation of amino acid residues. b) Zoom in on labelled MCL-1 peak, showing masses +16 Da, indicating oxidation of amino acid residues.
Peptide mapping of RTA-labelled MCL-1

To a solution of MCL-1 (50 μM) in ammonium acetate buffer (50 mM, pH 7.5) was added Ru(II)(bpy)₃-NOXA-B₁ (final concentration 10 μM), RTA ₆ (final concentration 10 μM) and APS (final concentration 10 μM) and the mixture was incubated at r.t. for 5 min. The mixture was irradiated for 1 min at r.t., 5 cm from light source (Kessil H150W-BLUE LED, 32W, 2 × lamps) and immediately quenched by the addition of DTT (final concentration 10 mM). The sample was split into two 50 μL aliquots. To each aliquot, a protease solution (Trypsin or Glu-C; Promega (Madison, WI); 20 ng μL⁻¹ in 25 mM ammonium bicarbonate) was added in a 1:50 ratio (protease:total protein content). Samples were incubated at 37 °C with shaking for 18 h. The digest reaction was stopped by adding 5 μL of 1% HCOOH, then subjected to purification using a Sep-pak 18 column. The Sep-pak column was equilibrated with 1 mL 0.1% TFA. 500 μL of 0.1% TFA was added to the peptide digest, the mixture was passed through the column and the column was washed with 1 mL 0.1% TFA. Peptides were eluted from the column with 500 μL MeCN-H₂O 1:1 + 0.1% HCOOH. The eluant was dried by vacuum centrifugation and the peptides were reconstituted in 20 μL 0.1% TFA. LC separation of the peptide mixtures was performed on an ACQUITY M-Class UPLC (Waters UK, Manchester). 1 μL sample was loaded onto a Symmetry C18 trap column and washed with 1% MeCN/0.1% HCOOH for 5 min at 5 μL min⁻¹, then the peptides were separated on a HSS T3 C18 analytical column (Waters UK, Manchester) by gradient elution of 1-60% solvent B (0.1% HCOOH in MeCN) in A (0.1% HCOOH in H₂O) over 30 min at 0.3 μL min⁻¹. The column eluant was directly interfaced to a quadrupole-orthogonal time of flight mass spectrometer (Xevo G2-XS Q-TOF, Waters UK, Manchester) via a Z-spray nanoflow electrospray source. The MS was operated in positive TOF mode using a capillary voltage of 3.0 kV, cone voltage of 40 V, source offset of 80 V, backing pressure of 3.58 mbar. The source temperature was 80 °C. Argon was used as the buffer gas at a pressure of 8.6 × 10⁻³ mbar in the trap and transfer regions. Mass calibration was performed using [Glu]-fibrinopeptide (GFP) at a concentration of 250 fmol μL⁻¹. GFP was also used as a lock mass calibrant with a one second lock spray scan taken every 30 s during acquisition. Ten scans were averaged to determine the lock mass correction factor. Data acquisition was using data dependent analysis with a 0.2 s scan MS over m/z 350-2000 being followed by five 0.5 s MS/MS taken of the five most intense ions in the MS spectrum. CE applied was dependent upon charge state and mass of the ion selected. Dynamic exclusion of 60 s was used. Data processing was performed using the MassLynx v4.1 suite of software supplied with the mass spectrometer. Peptide MS/MS data were processed with PEAKS Studio (Bioinformatic Solutions Inc, Waterloo, Ontario, Canada) and searched against the amino acid sequence. 176.0951 Da was set as a variable modification on any residues to
determine the position of the RTA 6 modification. MS mass tolerance was 10 ppm, and fragment ion mass tolerance was 0.05 Da. The false discovery rate was set to 1%.

Figure S21: Peptide fragments (shown in blue) detected by LC-MS/MS upon digestion of unmodified MCL-1 using Glu-C and trypsin proteases. Sequence coverage 97%.
Figure S22: Peptide fragments (shown in blue) detected by LC-MS/MS upon digestion of MCL-1 modified with RTA 6, using Glu-C and trypsin proteases. Sequence coverage 94%. ‘h’ denotes the position of the additional mass of 176.10 Da on the modified peptides.

Figure S23: Q-TOF MS/MS spectrum for a selected peptide modified with minimal RTA 6. Observed y and b ions are shown in red and blue, respectively. Modification is found on residue Cys286.
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