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Selective protein unfolding: A universal mechanism of action for the development of irreversible inhibitors

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In this work we screened a unique open access compound library on four structurally unrelated protein targets (Figure 1) in order to identify protein specific hits. The library consisted of 940 compounds (Open Collection (Scaffolds)),[1] a subset (139 compounds) was obtained from Australian natural sources, such as endophytic fungi,[2] macro-fungi,[3] plants,[4] and marine invertebrates.[5, 6] Approximately 15% of the library compounds were semi-synthetic natural product analogues,[3, 7, 8] while a small percentage (~5%) are known commercial drugs or synthetic compounds inspired by natural products.

Acknowledgements

We thank Compounds Australia (www.griffith.edu.au/science-aviation/compounds-australia) for access to the Davis open-access natural product library, which forms part of the Open Access Compound Collection.

ESI

Materials and Methods

Protein targets

GFP-AviTag, Ec BirA-GFP, Bp BirA-GFP, Bp DnaB-GFP, Bp DnaG-GFP and RdRp-GFP were expressed and purified as previously described.[9-12] All proteins were quantified by Bradford assay. BirA-GFP were resuspended in BirA buffer (25 mM Tris (pH 8), 100 mM NaCl, 5% v/v glycerol). GFP-AviTag, DnaB-GFP, DnaG-GFP and RdRp-GFP were resuspended in phosphate buffer (45 mM Na_2HPO_4, 5 mM NaH_2PO_4, 10% v/v glycerol, 2 mM β-mercaptoethanol).

The Mt BirA sequence (UniProtKB entry code: P96884) was codon optimized (Bioneer) for optimal expression in *E.coli*, and cloned into pIM013 (pET-uvGFP)[13] to create pAC286 (pET-N-6HIS-MtbBirA-GFP-C). The Mt BirA-GFP was expressed in *E. coli* BL21(DE3)RIPL using Overnight Express TB Medium (Novagen) containing 100 µg/ml ampicillin as previously described.[14] The medium (100 ml in a 1 L conical flask) was inoculated with 1 mL of an overnight starter culture and incubated at 37°C with shaking at 200 rpm until the optical density reached 0.5. The culture was then incubated for ~ 3 days at 16°C. Lysis and purification procedures were performed as previously described for Ec BirA-GFP.[11] Protein concentration was determined by Bradford Assay and protein purity by SDS-PAGE.

Compound library

Compounds for protein stability screening were sourced from Compounds Australia, Griffith University (www.griffith.edu.au/science-aviation/compounds-australia).[1] All compounds were supplied as 5 mM stock solutions in DMSO. A subset of compounds were accessed from a natural product-based open-access library that was created in

2010 by R.A.D and at the time of screening consisted of distinct small molecules, the majority of which were isolated from Australian plants, marine invertebrates, or endophytic fungi that are archived at the Griffith Institute for Drug Discovery, Griffith University, Australia. Several library compounds have been purchased from Sigma-Aldrich and PhytoLab. All compounds were analyzed for purity prior to screening and were shown by LC-MS or ^{1}H NMR analysis to have purities of >95%.

Compound screens with HT DSF-GTP

Ec BirA-GFP, Bp DnaB-GFP, Bp DnaG-GFP or RdRp-GFP were diluted to 1 μM in their respective buffers. Proteins (49 μl at 1 μM) were mixed with each compound (1 μl at 5 mM in DMSO) in separate wells of a 96-well clear PCR plate (Biorad, HSP9601). One well containing only DMSO and one blank well were included as controls. Reactions were incubated for 10 min at RT then heated in a real-time thermal cycler (IQ5 iCycler, Bio-Rad) from 30ºC-85ºC, increasing in 0.5ºC increments every 30 s. Data was analysed as previously described.[15] 94 compound scaffolds of interest were selected from the initial screen and repeated as above for all four proteins.

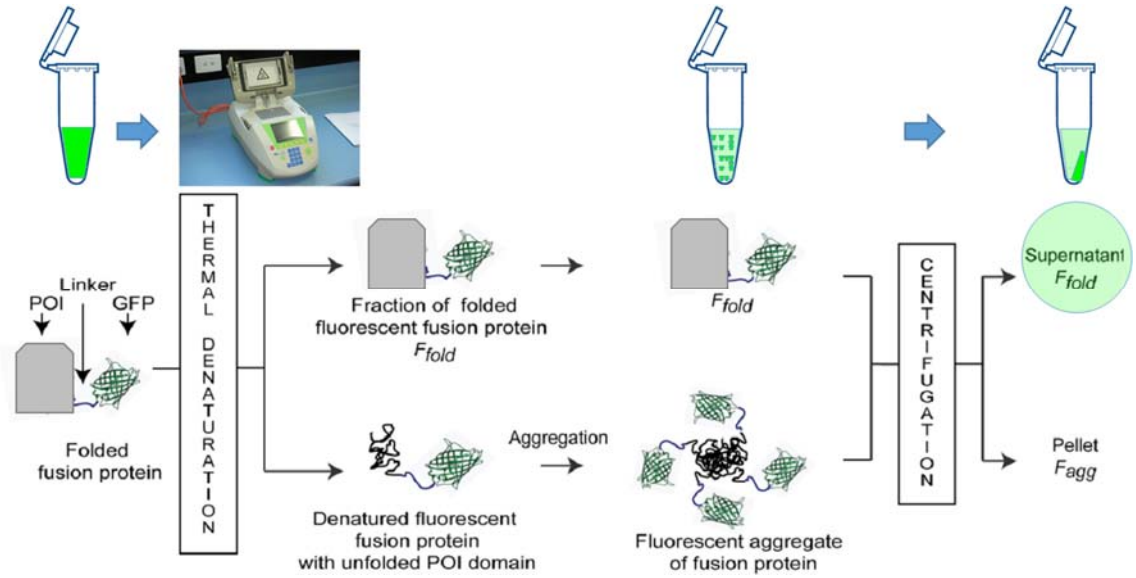
For concentration dependence studies with 11 selected compounds (Compounds Australia), Ec BirA-GFP (98 μl at 1 μM in BirA buffer) was mixed with each compound (2 μl at 78 μM-5 mM in DMSO). For SAR studies, 10 variants of 8 compound scaffolds (1 μl at 5 mM in DMSO) selected from the initial screen (Compounds Australia) were mixed with Ec BirA-GFP (49 μl at 1 μM in BirA buffer).

For concentration dependence and SAR studies with lovastatin, pravastatin, simvastatin, fluvastatin (Sigma Aldrich) and altenusin (Enzo Life Sciences), Ec, Bp, or Mt BirA-GFP (98 μl at 1 μM in BirA/2 buffer (25 mM Tris (pH 8), 50 mM NaCl, 2.5%

v/v glycerol) was mixed with each compound (2 µl at 78 µM-5 mM in DMSO). Melt-curve protocol was set from 25-85°C at 0.5°C/30 s. Reactions were run in duplicate.

Isothermal GFP-Basta

Reactions consisted of 49 µl Ec BirA-GFP (1 µM in BirA/2 buffer supplemented with ATP (1 µM), $MgCl_2$ (1 µM) and biotin (1 µM)) and 1 µl of either lovastatin, fluvastatin, pravastatin or simvastatin (5 mM in DMSO). Reactions were incubated at 37 ºC for 0, 10 and 30 min in a thermal cycler (Schematic 1). Following incubation, reactions were placed on ice for 5 min, then centrifuged at 18,000 *g* for 10 min at 4ºC. 25 µl of the supernatant of each reaction was combined with 40 µl Tris (25 mM, ph 8) in black 96-well flat bottom microtitre plates (Nunc), and the fluorescent F_{fold} was determined with a fluorescence plate reader (Victor V Wallace Perkin-Elmer). The excitation and emission filters were set at 355 nm and 535 nm, respectively, with 40 nm band-width. Data were normalized against the fluorescence of an untreated sample as described previously.[13]



Schematic 1: Principle of GFP-Basta. POI: protein of interest (i.e. BirA)

Inhibition of BirA biotinylation activity

A volume of 1 µl Ec BirA-GFP (15 µM in BirA buffer) was mixed with 18 µl of 25 mM Tris (pH 8) and 1 µl of DMSO containing a statin (10 mM), or altenusin (2 mM). Control reactions without the addition of DMSO or compounds were also included. Reactions were incubated at 25 or 37 ºC for 60 min in a thermal cycler.

Following incubation, reactions were mixed with 4 µl GFP AviTag (375 µM in phosphate buffer)[11] and 26 µl biotinylation premix (0.96 mM biotin, 0.48 mM ATP, 1.92 mM $MgCl_2$). Control reactions without GFP-AviTag and biotinylation buffer without BirA were also included. Reactions were incubated for a further 60 min at 37 ºC.

Unreacted biotin was then removed from the reaction using Profinity IMAC Nickel resin (Biorad) and Micro-Bio Spin Columns (Biorad) as previously described[11]. Reactions were bound to 50 µl pre-equilibrated nickel resin slurry for 5 min at 4ºC, then passed through the resin twice by centrifugation at 1000x *g* for 1 min at 4°C. The resin was washed twice with 500 µl of BirA buffer by centrifugation at 1000x *g* for 1 min at 4°C. The proteins were eluted from the spin column with 75 µl of the same buffer supplemented with 200 mM imidazole and centrifugation at 1,000x *g* for 1 min at 4°C following 5 min incubation at 4°C. The eluate was then passed a second time through the same spin column as for first elution. All control reactions were processed in identical fashion for comparison to account for protein losses during purification and reaction steps and for normalization.

Affinity purified biotinylated GFP-AviTag (10 µl) was incubated with 1 µl Stv (5 mg/ml, Invitrogen) at room temperature for 15 min. Control reactions containing non-biotinylated GFP-AviTag were not incubated with streptavidin. Reactions (10 µl) were mixed with non-reducing 2x SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS,

10% glycerol, 0.01% bromophenol blue), then subjected to 10% SDS-PAGE. Biotinylation and inhibition of biotinylation by statins were detected by UV exposure of the gel post-migration as previously described.[11]

Target access in lysate and serum

A single colony of BL21(DE3)RIPL was inoculated in 50 ml LB and incubated at 37°C and 200 rpm. Bacteria were harvested at stationary phase by centrifugation at 2800 g for 10 min. The supernatant was discarded and the cells were resuspended in ice cold BirA Buffer at 7 ml/g cell pellet. Cells were lysed by passage through an ice cold French pressure cell at 12,000 psi. Insoluble debris was removed by centrifugation at 40,000g for 40 min at 4°C. Lysate was heat treated at 95°C for 5 min and clarified by centrifugation at 18,000 g at 4°C for 10 min if required.

Human serum (Invitrogen), was diluted 1/4 in BirA buffer. If required, diluted serum was heat treated and clarified identically to the bacterial lysate.

DSF-GTP reactions consisted of 5 µl of Ec BirA-GFP (10 µM in BirA buffer), 19 µl of either lysate or diluted serum (i.e. untreated, denatured, or denatured and clarified), 1 µl of test compound (5 mM in DMSO) and 25 µl ddH_2O. For control reactions, test compounds were replaced with DMSO. Melt-curve protocol was set from 25-85°C at 0.5°C/30 s. Reactions were run in duplicate.

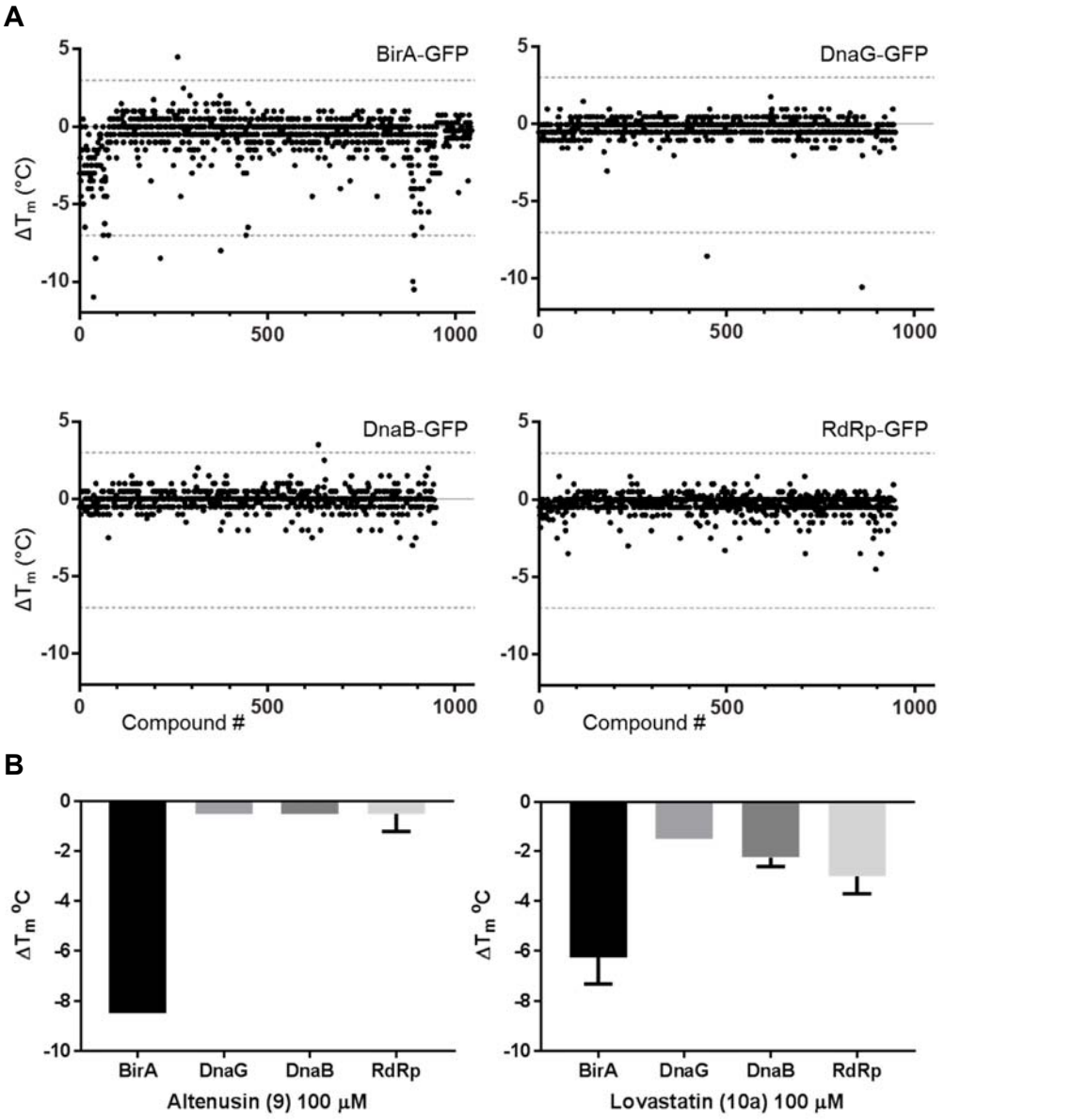


Figure S1 A) HT DSF-GTP screening of 940 pure compounds with Ec BirA-GFP, DnaG-GFP, DnaB-GFP and RdRp-GFP (ESI for detailed methods). Compounds (1 µL at 5 mM in DMSO, Compounds Australia) were dispensed in 96-well clear PCR plates (Biorad, HSP9601). Target-GFP (49 µL at 1 µM) were added to each compound for 15 min at RT prior subjecting the reactions to a melt-curve protocol (30-85°C at 0.5°C/30 s) in a IQ5 iCycler (Bio-Rad). See ESI for buffer composition. T_m were analyzed as previously described.[15] B) Destabilizing effect of altenusin **9** and lovastatin **10a** on Ec BirA-, DnaG-, DnaB- and RdRp-GFP. Error bars represent SD (N=2).

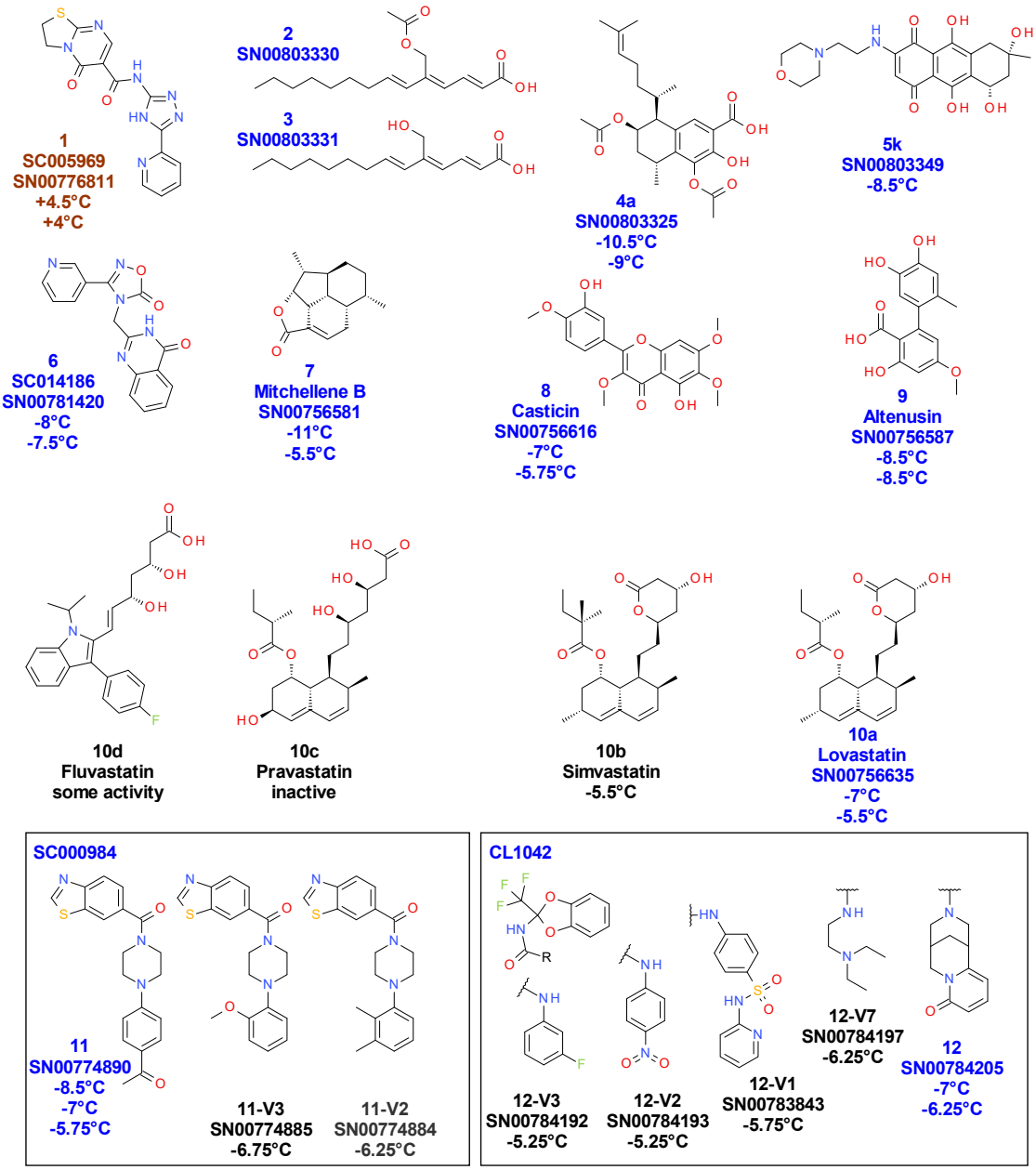


Figure S2A: Selected compounds and their effect on Ec BirA-GFP T_m. ΔT_m values are indicated. Hits from first screen are labelled in blue and brown. SN library identification codes as well as SC and CL scaffold codes are indicated. Boxed areas display the most active compounds identified in the subsequent SAR scaffolds screen SC000984 (see Fig. 3 and S3) and CL1042 (see Fig. S3). The structures, properties and SMILES of listed compounds can be retrieved via CASPeR using their SN codes (Compound Australia Library https://gc-prd-eskitis.rcs.griffith.edu.au/casper/web/). Independently repeated T_m values are indicated below first screen values.

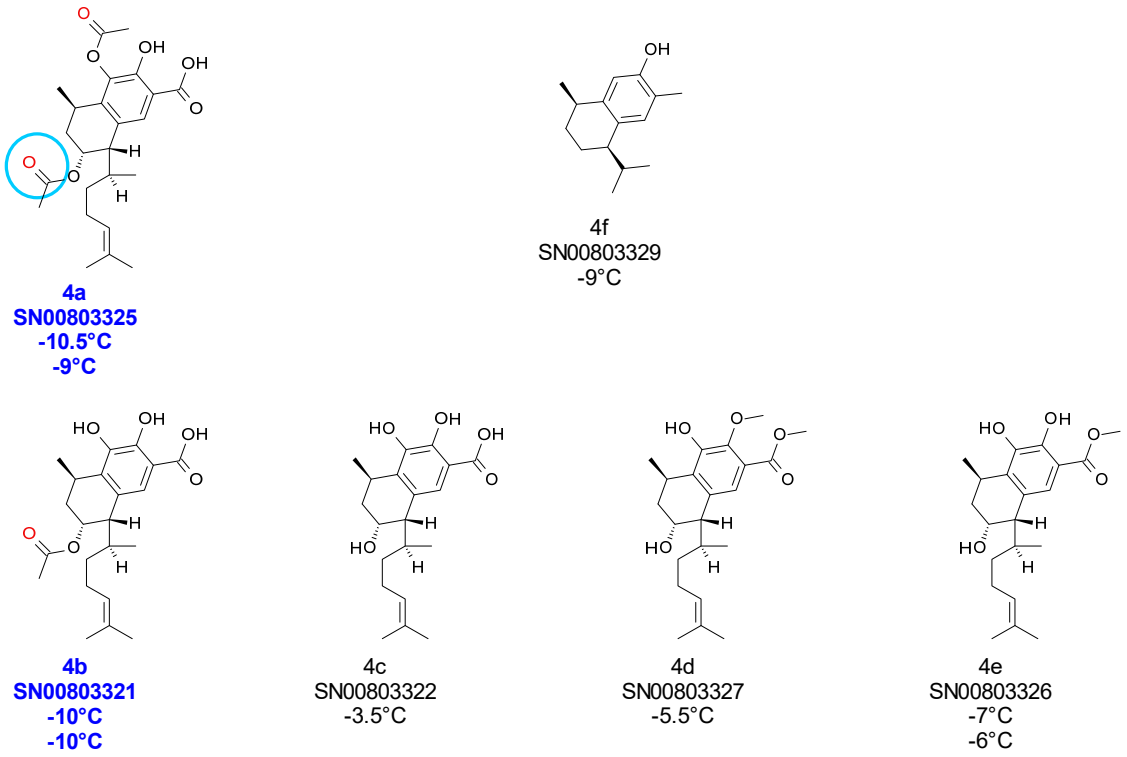


Figure S2B: Serrulatane analogues **4a-e** and minimal structure **4f** and their destabilising effect on Ec BirA-GFP T_m. ΔT_m values are indicated. SN library identification codes are indicated. The structures, properties and SMILES of listed compounds can be retrieved via CASPeR using their SN codes (Compound Australia Library https://gc-prd-eskitis.rcs.griffith.edu.au/casper/web/). Independently repeated T_m values are indicated below first screen values.

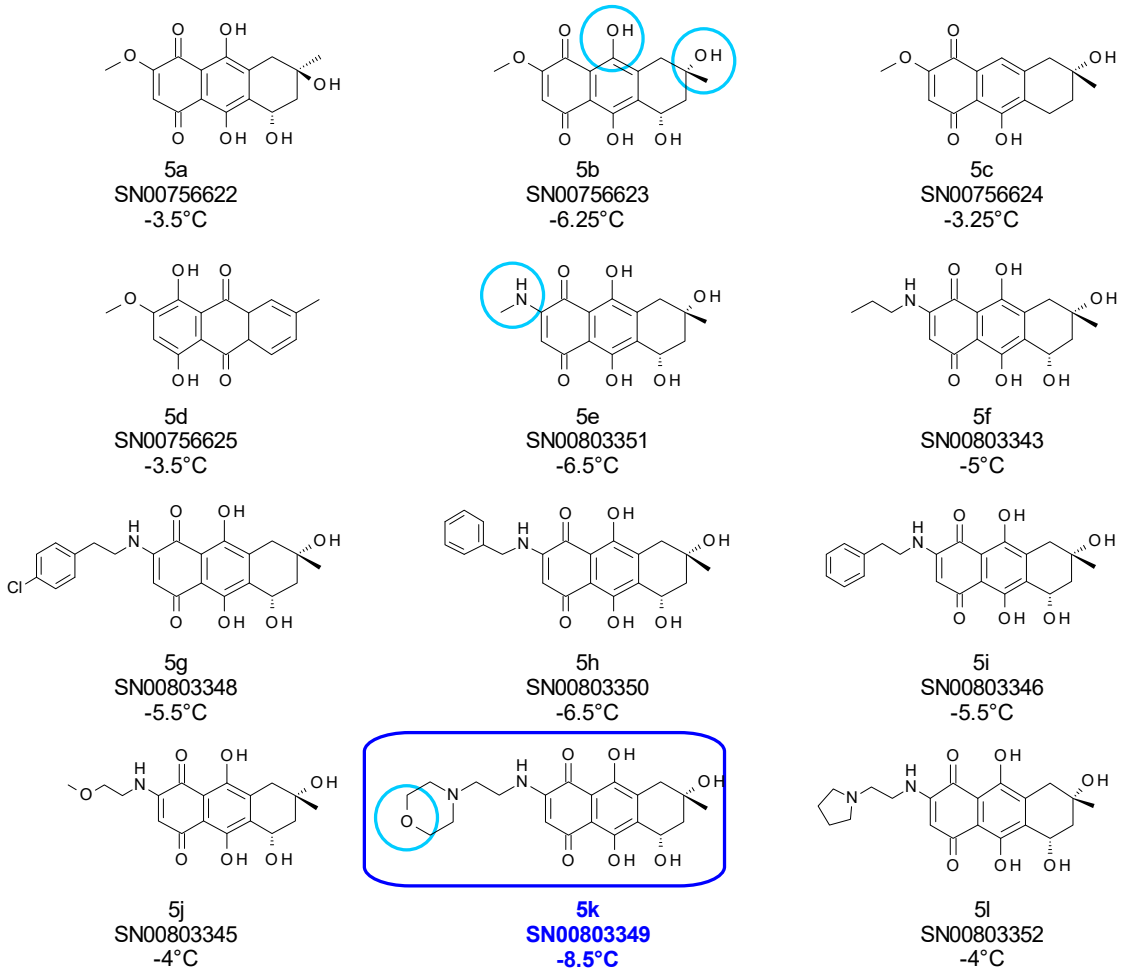


Figure S2C: Tetrahydroanthraquinone analogues and their destabilising effect on Ec BirA-GFP T_m. ΔT_m values are indicated. SN library identification codes are indicated. The structures, properties and SMILES of listed compounds can be retrieved via CASPeR using their SN codes (https://gc-prd-eskitis.rcs.griffith.edu.au/casper/web/).

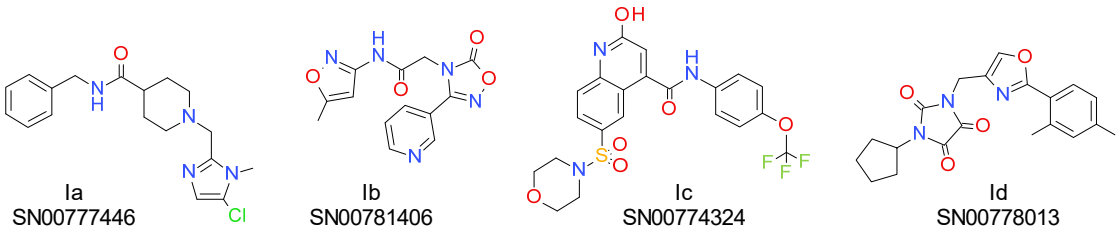


Figure S2D: Inactive compounds. SN library identification codes are indicated. The structures, properties and SMILES of listed compounds can be retrieved via CASPeR using their SN codes (https://gc-prd-eskitis.rcs.griffith.edu.au/casper/web/).

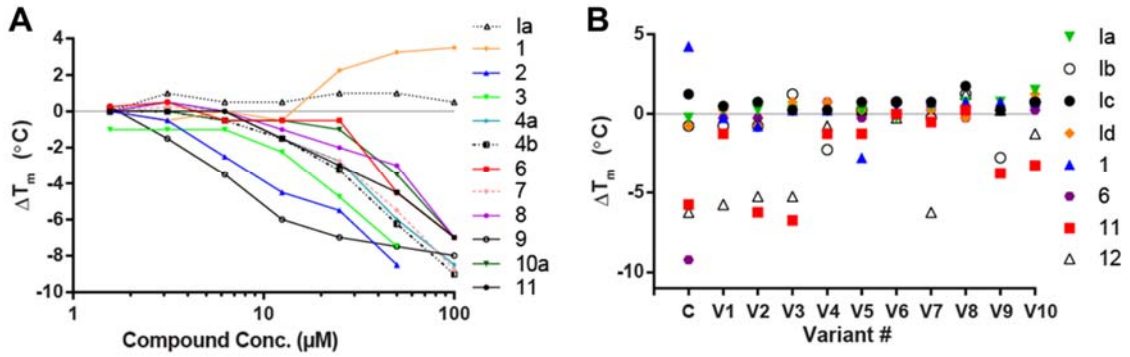


Figure S3. Concentration dependence (A) and SAR studies (B). A) Effect of two-fold serially diluted compounds on Ec BirA-GFP T_m. One inactive compound **Ia** and eleven hits were selected from the initial screen. B) Effect of structural analogues of four inactive compound **Ia-d** and four hits on Ec BirA-GFP T_m. For each inactive compound and hit (C: **Ia-d** and **1, 6, 11-12** as depicted), ten analogues (**V1-V10**) were tested (all at 100 μM). See Figure S2A and S2D for representative structures, SN and scaffold codes (Open Collection Scaffolds Library) and T_m values of the active variants of **11** (**11-V2** and **V3**) and **12** (**12-V1-3** and **V7**).

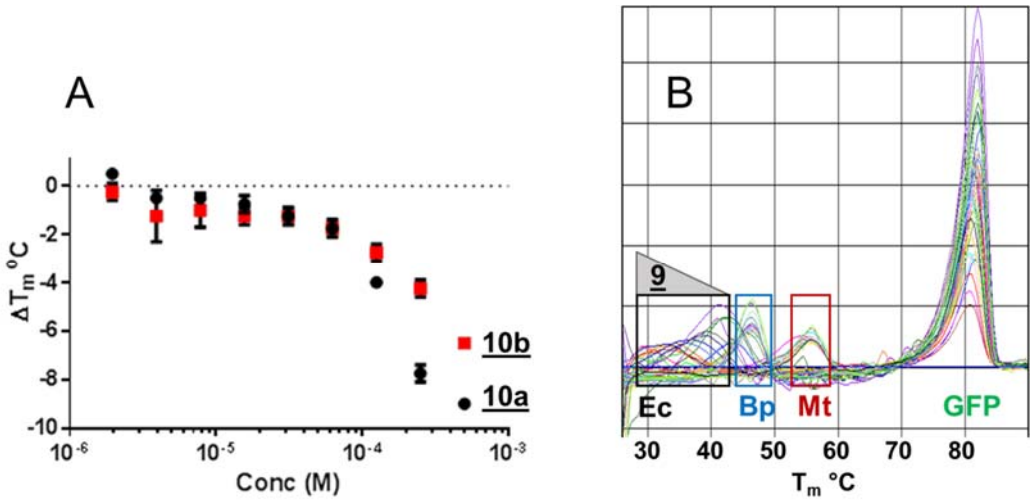
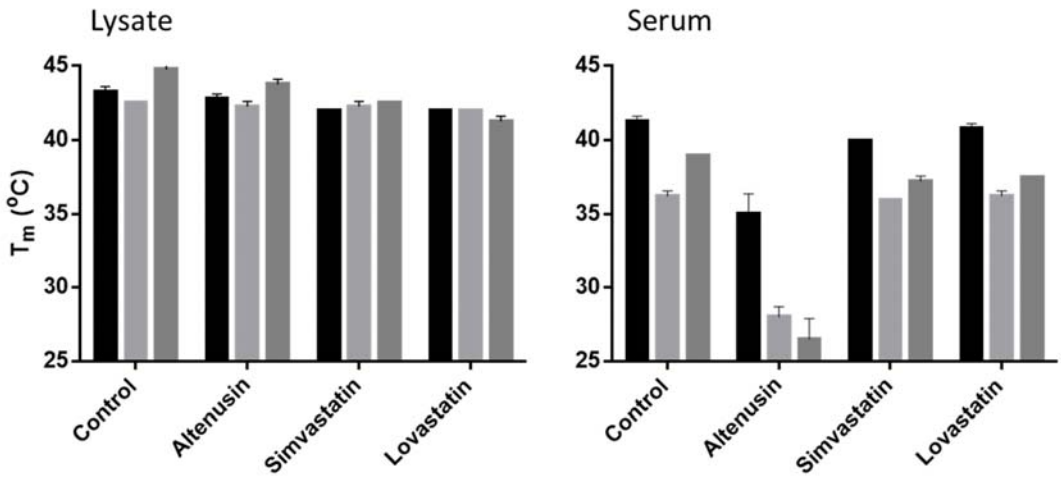


Fig. S4 A) SAR and concentration dependence of **10a** (black circles) and **10b** (red squares) on T_m of Bp BirA-GFP. Error bars represent SD (N=2). See Fig. 4 for comparison with Ec BirA-GFP. B) Concentration dependence of **9** on T_m of Ec, Bp, Mt BirA-GFP, and GFP. T_m peaks are shifting towards lower temperatures for Ec BirA-GFP with increasing concentrations of **9** (cf Fig. 6A). All other melt-curves are mostly unaffected. Melt-curves are superimposed (y-axis: dRFU/dT). Melt-curve protocol: 25-85°C at 0.5°C/30 s.



		T_m (°C)		
		untreated	95°C	95°C + clarified
Lysate	Control	43.3	42.5	44.8
	Altenusin	42.8	42.3	43.8
	Simvastatin	42.0	42.3	42.5
	Lovastatin	42.0	42.0	41.3
Serum	Control	41.3	36.3	39.0
	Altenusin	35.0	28.0	26.5
	Simvastatin	40.0	36.0	37.3
	Lovastatin	40.8	36.3	37.5

Figure S5. Effect of altenusin **9,** lovastatin **10a** and simvastatin **10b** (all at 100 μM) on *Ec* BirA-GFP T_m in the presence of Ec lysate or human serum. Data was obtained using DSF-GTP. Melt-curve protocol: 25-85°C at 0.5°C/30 s. Error bars on the graphs represent the SD between repeat reactions (N=2).

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