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Quantitative Chemoproteomic Profiling Reveals Multiple Target Interactions of Spongiolactone Derivatives in Leukemia Cells

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The spongiolactones are marine natural products with an unusual rearranged spongiane skeleton and a fused β-lactone ring. These compounds have potential anticancer properties, but their mode of action has yet to be explored. Here we employ activity-based protein profiling to identify the targets of a more potent spongiolactone derivative in live cancer cells, and compare these to the target profile of a simpler β-lactone. These proteomic hits provide the first insights into the covalent mechanism of action of this natural product class.

The spongiolactones are a class of natural products with anti-proliferative and potentially immunosuppressive properties.[1] These compounds contain a unique cyclopentane fused β-lactone, a potentially protein-reactive moiety targeting serine hydrolase active site nucleophiles in a range of cell types from cancer cells to bacteria.[2-4] However, the spongiolactone mechanism of action is yet to be elucidated. Previously we reported the first total synthesis of (+)-spongiolactone (**1**) and the discovery of a more potent, isomeric derivative, regio ($\Delta^{9,12}$), C6, C15-bis-epi-spongiolactone (±)-**2** (Fig. 1a).[5] This derivative, even as a racemate, exhibited greater potency toward a human chronic mylogenous leukemia cell line (K562) in comparison to the natural product (IC_{50} 29 ± 4 vs. 129 ± 10 μM, respectively). To further understand its mechanism of action, including identification of possible cellular targets, we developed an enantioselective synthesis of the isomeric spongiolactone derivative (+)-**2** in addition to a derived alkynyl probe (+)-**3.** This probe, along with the racemic version (±)-**3**, was then applied in activity-based protein profiling (ABPP)[6, 7] to elucidate the targets of this natural product derivative in live cells (Fig. 1b).

Based on our previously established synthetic route to spongiolactone, we utilized a kinetic resolution of a previously described advanced, racemic intermediate, tri-substituted cyclohexanone (±)-**8**, obtained in 3 steps from 1,3-cyclohexanedione (Scheme 1).[5] Under standard CBS reduction conditions the corresponding alcohol (+)-**9** was isolated in 37% yield[8, 9] (92% ee). The relative stereochemistry of the tetra-substituted cyclohexane (+)-**9** was assigned based on coupling constant analysis while the absolute stereochemistry was assigned based on the CBS model (see ESI for details).[10] Subsequent Swern oxidation delivered the optically active

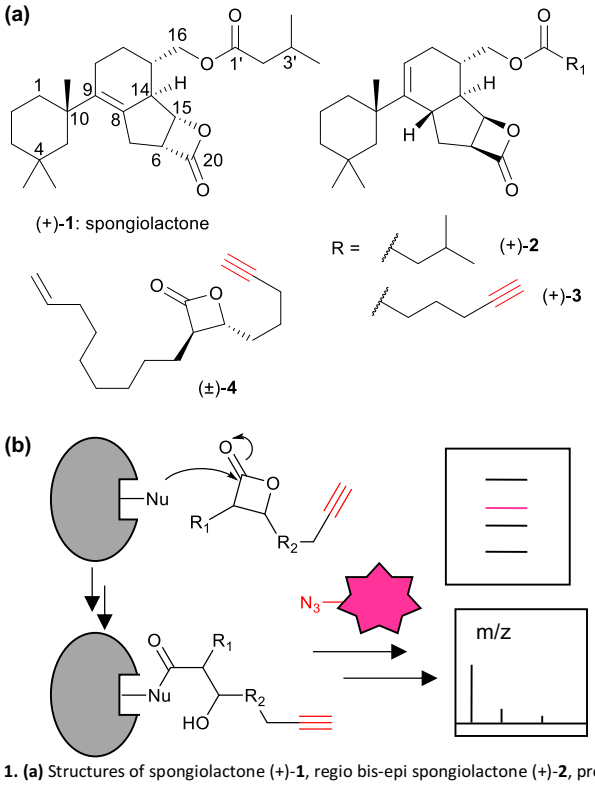

Fig. 1. (a) Structures of spongiolactone (+)-**1**, regio bis-epi spongiolactone (+)-**2**, probe (+)-**3**, and previous β-lactone probe (±)-**4**. **(b)** β-lactone containing compounds potentially react with active site nucleophiles on proteins; compounds containing an alkyne can be clicked to functionalized azides for ABPP experiments to identify protein targets via fluorescent SDS-PAGE or mass spectrometry.

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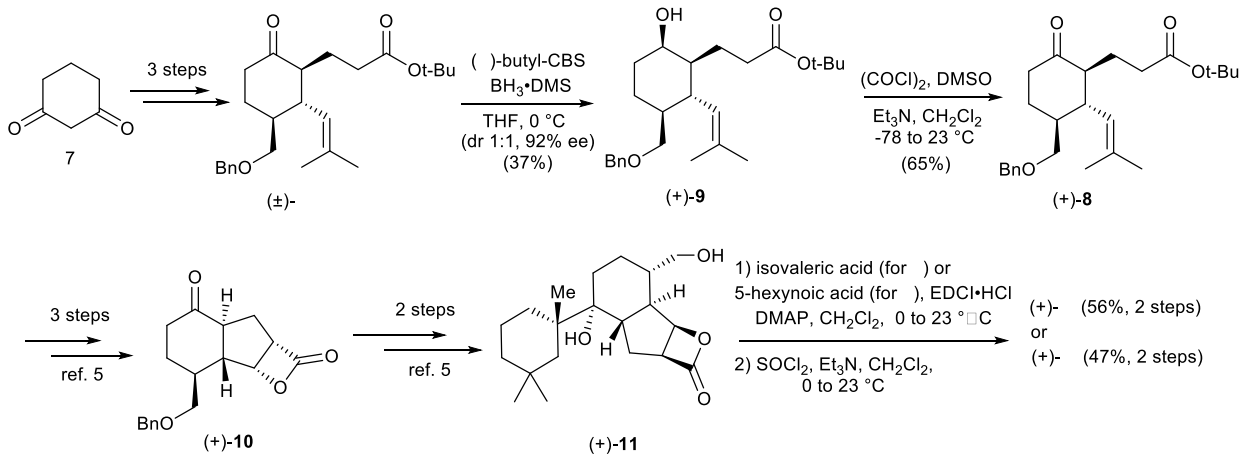

Scheme 1. Synthesis of compound (+)-**2** and probe (+)-**3**. See also Scheme S1 in Supplementary Information.

cyclohexanone (+)-**8**, previously described in racemic form. This cyclohexanone was then converted to primary alcohol (+)-**11** employing our previously established synthetic route via the tricyclic β-lactone (+)-**10** to deliver both regio, bis-epi spongiolactone (+)-**2** and the alkynyl probe (+)-**3** through esterification with isovaleric acid and 5-hexynoic acid, respectively. The racemic version of the alkynyl probe, (±)-**3** was prepared for initial protein profiling in an analogous fashion to the optically active probe (+)-**3** (Scheme S1).

Both parent compound (±)-**2** and probes (±)-**3** and (+)-**3** exhibited micromolar activity (14-30 μM) in a cell cytotoxicity assay against K562 cells and comparable activity in another leukemic cell line, Jurkat (Fig. 2a, Fig. S1). Enantiomerically pure (+)-**2** showed an increase in potency (~2-fold) compared to the racemic compound. Simple racemic, β-lactone (±)-**4**, which was previously utilized to label enzymes in bacteria,[2, 3] also showed growth inhibition of K562 cells, which was weaker but nonetheless in the same range as the spongiolactones.

We next sought to determine whether these β-lactone-containing compounds covalently labelled proteins in live cancer cells. The terminal alkyne handle of probe **3** enables downstream capture of probe-labelled proteins by copper-catalysed click chemistry (CuAAC) and analysis by in-gel fluorescence and proteomics (Fig. 1b).[11] In initial studies, K562 cells were incubated with 10 μM racemic probe (±)-**3** (or vehicle, DMSO, control) to obtain a global and absolute stereochemistry-independent labeling pattern. Cells were lysed and proteins ligated to rhodamine-azide (RhN_3) via CuAAC. Gel separation and fluorescence imaging revealed multiple bands, which were dose-dependently outcompeted by pre-incubation of cells with racemic regio bis-epi spongiolactone (±)-**2** and, to a lesser extent, spongiolactone (+)-**1** (Fig. S2), suggesting that the probe (±)-**3** labels the same proteins as spongiolactone and regio, bis-epi-spongiolactone. Enantiomerically pure probe (+)-**3** gave a similar pattern to racemic (±)-**3** in both K562 and Jurkat cells (Fig. 2b), with dose-dependent labelling down to 3 μM (Fig. S2). To identify labelled proteins, samples from K562 cells incubated with 10 μM (+)-**3** were ligated to biotin-N_3, enriched on avidin beads, digested by trypsin and peptides analysed by gel-free LC-MS/MS. Label free quantification (MaxLFQ[12]) was performed to quantify intensities and compare DMSO with probe-incubated samples. We observed high reproducibility across four biological replicates (Fig. S3). Consistent with the large numbers of bands on-gel, 104 proteins were significantly enriched in samples treated with (+)-**3** over controls (log_2(probe **3**/DMSO)>2 and *t*-test, Fig. 2d, Table S1). Gene Ontology (GO)[13] analysis revealed that hits are typically involved in lipid metabolism and transport processes (Table

(a)

	IC_{50} K562 (μM)	IC_{50} Jurkat (μM)
(+)-**2**	15.5 (0.9)	14.6 (0.4)
(+)-**3**	30.2 (1.4)	20.6*
(+)-**4**	n.d.	38.8 (3.0)

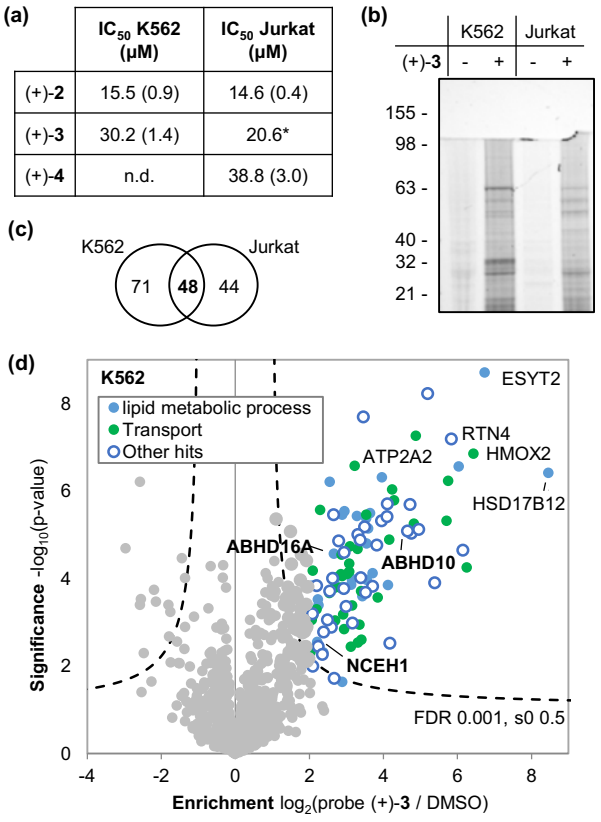

Fig. 2. (a) Cytotoxicity of compounds in K562 and Jurkat cells (MTT assay). Biological triplicate, except for * (single experiment, technical triplicate). n.d. = not determined. Weighted mean (error). See Supp. Fig. S1 for curves. **(b)** In-gel fluorescence analysis after labelling with (+)-**3** in K562 and Jurkat cells. **(c)** Overlap of hits in K562 and Jurkat cell lines. **(d)** Volcano plot depicting enrichment and significance of enrichment in chemical proteomics experiments with K562 cells (10 μM (+)-**3**). See Tables S1 and S2.

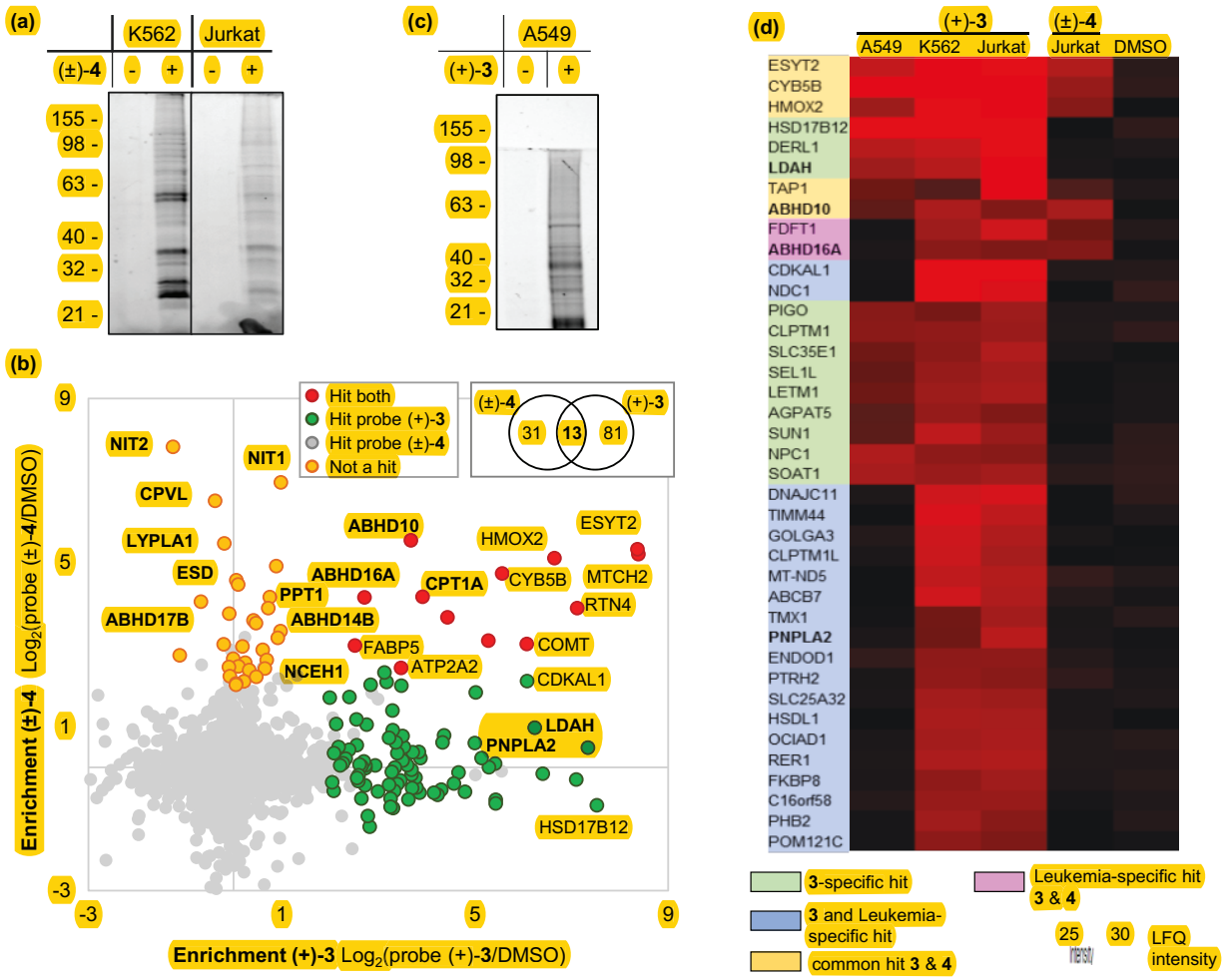

Fig. 3. (a) Gel-based analysis after labelling with 10 μM probe (±)-**4** in K562 and Jurkat cells. **(b)** Overlap of proteomic hits with probes (+)-**3** and (±)-**4** in Jurkat cells. Inset: Venn diagram overlap. Hits here were defined based on significance in the *t*-tests (Table S3). **Bold** = hydrolase. **(c)** Gel-based analysis of (+)-**3** labelling in A549 cells. **(d)** Heat-map analysis of LFQ intensities (mean across replicates) for proteins defined as hits in K562 and Jurkat cells. **Bold** = hydrolase. See Table S4.

S1). Predicted endoplasmic reticulum (ER) and mitochondria-localised membrane proteins dominated. Indeed, biochemical fractionation and gel analysis confirmed that many of the hits localised to the insoluble fraction (Fig. S2).

We repeated the chemical proteomic experiments with Jurkat cells; a large number of hits was again observed (Table S2, Fig. S6) with relatively high overlap between the two leukemic cell lines (Fig. 2c). GO enrichment analysis performed for the 48 high confidence overlapping proteins revealed that hits are preferentially involved in transport processes, lipid metabolism and localisation (Fig. S4). Surprisingly, only a few proteins with a predicted active site nucleophile (such as serine hydrolases) were present amongst the hits; these included mycophenolic acid esterase ABHD10, phosphatidylserine hydrolase ABHD16A,[14] and sterol hydrolase NCEH1. These data suggest that, at concentrations concurrent with cytotoxicity, probe (+)-**3** labels a more diverse set of proteins than hydrolases.

We next asked whether the simple β-lactone (±)-**4**, which binds hydrolases as well as other proteins in bacteria,[2, 3] labels a similar profile of targets to (+)-**3** in mammalian cells. Probe (±)-**4** labelled proteins in K562 cells in a dose-dependent manner (Fig. S5) and gave a similar profile in Jurkat cells (Fig. 3a). Interestingly, probe (±)-**4** exhibited a markedly distinct labelling pattern compared to the regio bis-epi spongiolactone-derived probe (+)-**3** (Fig. 3), suggesting that the scaffold in which the β-lactone warhead is embedded, not surprisingly, has a dramatic effect on preferences for protein binding partners. Chemical proteomic analysis of labelled proteins in Jurkats revealed that, in contrast to (+)-**3**, ~50% of (±)-**4** hits have annotated hydrolase activity (Fig. S5). GO analysis classifies many hits as proteins involved in lipid binding or metabolism (Table S3, Fig. S5). Thus many proteins labelled by (±)-**4** are lipid metabolic enzymes with hydrolase activity, as might be expected from the lipid-like structure of this probe (Fig. 1a).

Comparison with the data from labelling with (+)-**3** shows overlapping and distinct targets of the two probes in Jurkats (Fig. 3b, Table S3). In particular, it is clear that (±)-**4** labels many more hydrolases, but that some proteins non-hydrolases are labelled by both probes. These common hits include heme-binding, oxidoreductases HMOX2 and CYB5B, and membrane proteins with transport or structural roles in the ER and mitochondria (ESYT2, RTN4, MTCH2, TAP1, MT-ND5, AT2A2). The enrichment of these, mostly abundant, proteins may be

due to promiscuous reactivity of both probes in the membrane environment. However, interestingly, acrylamide covalent probes have recently been shown to selectively label a specific cysteine residue on RTN4, impairing cancer cell growth.[15] Common hydrolase hits of both probes include ABHD16A, a lipase important for phosphatidylserine metabolism that has been previously shown to be covalently labelled and inhibited by a β-lactone probe in K562 and brain cells.[14]

We previously observed (±)-**2** to be >4-fold more toxic towards leukemia cell line K562 than other cell lines.[5] Consistent with this, (+)-**2**, its corresponding probe (+)-**3,** as well as simple β-lactone (±)-**4**, all showed greatly reduced activity against lung cancer cell line A549 (>100 μM, Supp. Fig. S1). We hypothesised that such selective toxicity could be due to differences in either cellular targets, the susceptibility of different cell lines towards perturbation of specific pathways via the same targets, or compound metabolism, uptake or efflux. ABPP with the regio bis-epi spongiolactone-derived probe (+)-**3** in A549 cells gave a different pattern (Fig. 3c, S6) but still strong labelling, suggesting that the probe is efficiently taken up by A549 cells and labels proteins. Chemical proteomic analysis revealed moderate overlap of potential hits with the leukemia cell lines (Fig. S7, Table S4). We selected those proteins classified as strong hits in both K562 and Jurkats cells, and compared these across both probes and all cell lines via clustering of mean LFQ intensities (Fig. S7). Proteins that showed intensity in DMSO controls were removed to further refine the list down to high confidence binders (Fig. 3d).

This analysis revealed subsets of hits with differing cell line- and probe-specificity. For example, HMOX2 is a clear target in all contexts. This important heme homeostasis protein has multiple cysteines present in heme regulatory motifs that may react with small molecules.[16] In contrast, dehydrogenase enzyme HSD17B12 is a strong probe (+)-**3**-specific hit; HSD17B12 acts on very long chain fatty acyl chains as well as estrogen,[17, 18] and the fused ring scaffold of (+)-**3** may explain why it strongly labels this protein whilst the simple dialkyl β-lactone probe (±)-**4** does not. A large number of hits (22) appear to be labelled by (+)-**3** only in leukemia cells, with only two of these are also labelled by (±)-**4**. Overall these data suggest that interaction of (+)-**3** with multiple targets in K562 and Jurkat cells is likely responsible for its cancer cell cytotoxicity.

In conclusion, we synthesised a spongiolactone-derived probe and performed quantitative chemical proteomic profiling to identify the potential protein targets in live cells. Probe (+)-**3** based on regio, bis-epi-spongiolactone (+)-**2** addresses a broad array of targets involved in various pathways, suggesting that its bioactivity likely stems from polypharmacology. Comparison with the target profile of a simpler β-lactone adds to the growing body of evidence that the proteome reactivity of electrophilic moieties, not surprisingly, is heavily influenced by structural features surrounding the reactive β-lactone. Probe (±)-**4**, which mimics natural products such as lipstatin, preferentially labels hydrolases, whereas the spongiolactone-derived probe, embedded in a more rigid and complex scaffold, addresses a distinct spectrum of targets. This is an important observation given the continued exploitation of electrophilic natural products, and in particular β-lactones, as bioactive compounds and chemical probes.[11] Finally, the proteomic hits presented here are valuable starting points for further understanding the mode of action and differing potencies of spongiolactones in diverse cellular contexts.

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