CHEMICAL BIOLOGY

# Diverse engineering

Methods for generating molecular diversity provide a route to screen a wider section of chemical space, to discover compounds with useful biological properties. Now, a complexity-to-diversity strategy has enabled the discovery of a multi-cyclic structure from a complex natural product that induces ferroptotic cell death in cancer cells.

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Natural products and synthetic small molecules provide the means to dissect and manipulate biological processes with spatial and temporal resolution. The identification of biologically active small molecules by high-throughput screening remains limited by the lack of structural diversity and the restricted three-dimensional complexity of compounds that can be found within traditional chemical libraries. To address this, a number of strategies have been designed that produce collections of structurally diverse and complex small molecules[1]. For instance, such approaches have led to the development of new scaffolds that are not found in nature, the identification of chemical probes that can modulate protein functions in unique ways, and the discovery of potentially druggable targets[2]. Another valuable strategy to identify new biologically active compounds is to produce libraries of molecules inspired by complex natural product scaffolds[3]. Now, writing in *Nature Chemistry*, Paul Hergenrother and co-workers have established a powerful complexity-to-diversity strategy[4], which they use to fabricate small molecules with a diverse range of structures. These natural product-derived multi-cyclic structures can possess interesting and potent biological activity.

This method harnesses the structural complexity of existing natural products to generate molecular diversity through short synthetic sequences[5]. Chemical modification of the natural product pleuromutilin (**1**) led to the efficient synthesis of molecules containing novel complex ring architectures (Fig. 1a). Diterpene **1** is composed of a dense 5-, 6- and 8-membered ring system harbouring no less than eight contiguous stereogenic centres. Contraction of the 8-membered ring through a carbocation rearrangement led to the core structure of **2**, which gave rise to **3** via few additional synthetic steps including a lead-mediated ring expansion. In contrast, acid-mediated isomerization, together with a 1,5-hydride shift and expansion of the 6-membered ring

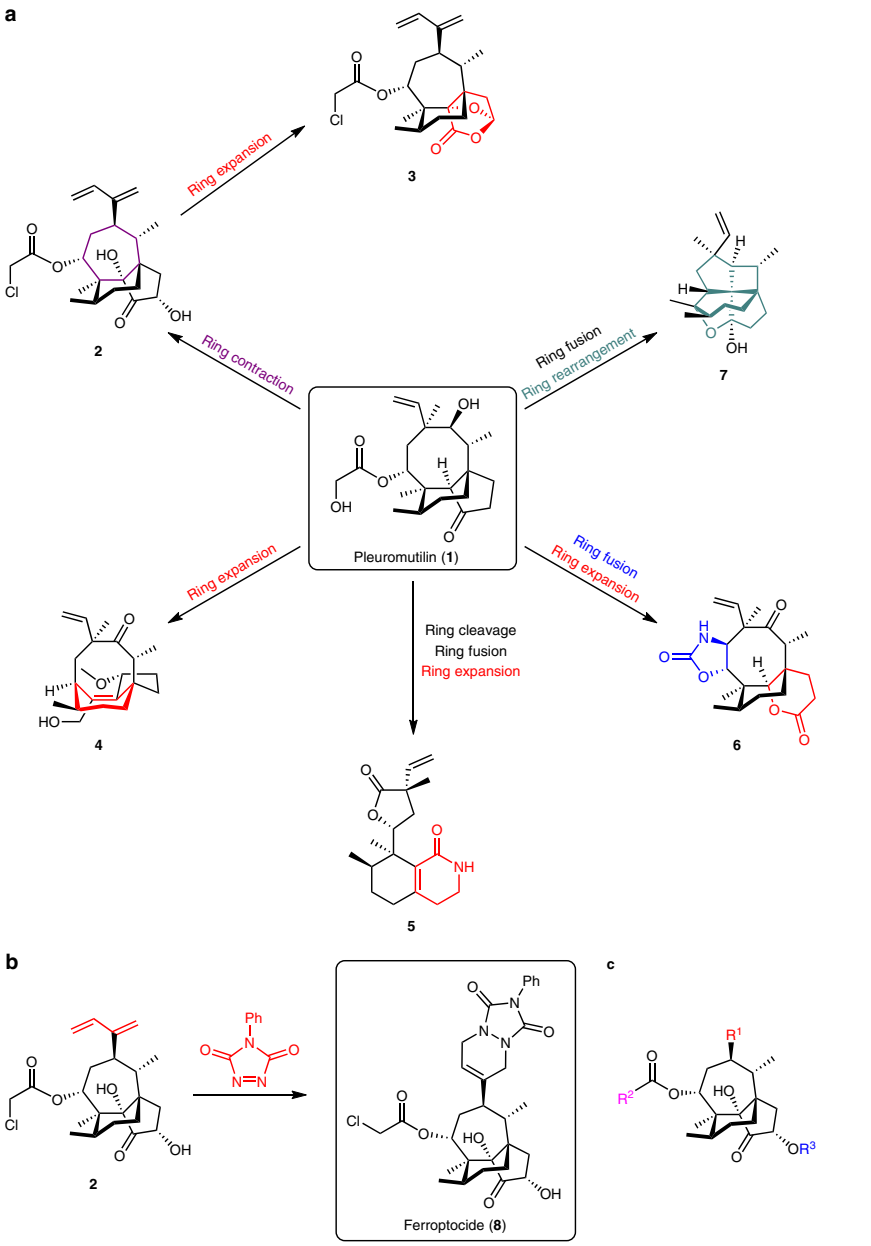


**Fig. 1 | Complexity-to-diversity strategy reveals a novel thioredoxin inhibitor and inducer of ferroptosis. a**, Diversity-generating processes illustrating conversions of the complex natural product pleuromutilin (**1**) into structurally distinct ring systems. **b**, Functionalization of a complex molecular framework through a [4+2] cycloaddition yields the biologically active small molecule ferroptocide (**8**). **c**, Additional appendage functionalization expands molecular diversity.

of **1**, afforded the complex core of **4**. Alternatively, a combination of ring cleavage through a retro-Michael reaction and ring expansion involving a Beckmann rearrangement afforded **5**. Complex polycyclic structures **6** and **7** were also both independently produced from **1** using distinct synthetic methodologies, illustrating the versatile nature of this strategy, which generated 12 structurally complex compounds with novel ring systems from a single natural product.

Evaluation of the biological properties showed **2** to be cytotoxic when tested against a panel of cancer cell lines. Intermediate **2** was then further diversified by taking advantage of available functional groups to provide a variety of additional modifications to the core structure (Fig. 1b, c). Functionalization led to the formation of the more potent compound **8**, named ferroptocide, along with other derivatives. Importantly, **8** showed potent cytotoxic properties against primary cancer cells freshly isolated from cancer patients, killing these cells more rapidly than conventional drugs used in the clinical management of cancer.

Comparing the effect of **8** with a series of cytotoxic drugs indicated an atypical profile. In particular, **8** induced cell death more rapidly compared to other pro-apoptotic agents, suggesting that **8** triggered a distinct cell death pathway. This was supported by the absence of PARP-1 cleavage and the lack of protective effect of a caspase inhibitor, thereby ruling out apoptosis. Morphological analysis using transmission electron microscopy showed mitochondrial defects, and confocal microscopy indicated that a fluorescent analogue of **8** co-localized with a mitochondrial marker. Importantly, treatment of cancer cells with **8** led to the production of reactive oxygen species (ROS) in mitochondria, providing solid evidence that **8** mediates its activity by directly targeting this organelle.

Additional mechanistic investigation indicated that **8** triggered a cell death pathway consistent with ferroptosis, a non-apoptotic form of cell death characterized by an iron-dependent ROS-mediated lipid peroxidation[6]. The onset of lipid-peroxidation upon treatment with **8** was monitored using C11-BODIPY staining. The ROS scavenger N-acetyl cysteine, the iron chelator deferoxamine and the ferroptosis inhibitor ferrostatin-1 reduced the effect of **8**, protecting cells from cell death and robustly establishing **8** as a novel inducer of ferroptosis. In contrast to other agents susceptible to elicit ferroptosis, **8** did not inhibit the ferroptosis regulator glutathione peroxidase **4** indicating a distinct mechanism of action. Affinity isolation using a biotin-labelled analogue combined with proteomic analysis identified putative targets of **8** including thioredoxin, a protein involved in redox signalling.

Attempts to knock out thioredoxin phenocopied the effect of **8**, and knocking down thioredoxin consistently led to the production of ROS, lipid peroxidation and sensitized cells to treatment with **8**, suggesting that thioredoxin is a functional target of **8**. Furthermore, **8** inhibited the activity of thioredoxin in vitro to a greater extent compared to known thioredoxin inhibitors. The need for an α-chloro ester to maintain the activity of **8** argued in favour of covalent binding with protein targets. In line with this, site-directed mutagenesis was employed to replace key cysteine residues with serine, which revealed that **8** can covalently react with two of these residues within the active site of thioredoxin, validating this protein as a target of **8**. Unlike other thioredoxin inhibitors, the ability of **8** to covalently react with thiol residues of thioredoxin enables the manipulation of thioredoxin in a distinct manner and the ability to further characterize the mechanisms underlying ferroptotic cell death.

This remarkable discovery-based study illustrates the power of synthetic organic chemistry to produce complex structures operating through unique mechanisms. Complexity-to-diversity can be implemented using virtually any complex natural product as starting material, representing a conceptually appealing approach; however, the underlying chemistry can be elaborated upon, making it a challenging endeavour. In contrast to in vitro and in silico screening approaches, which do not take into account the complexity of cellular systems, studies based on cell phenotypes can directly reveal biological targets and molecules with potent activity without requiring prior knowledge of their mechanism of action. The study from Hergenrother and co-workers combines complexity-to-diversity with an in-depth biological investigation to identify thioredoxin as mechanistic target of ferroptocide **8** — insight which could potentially lead to the development of distinct scaffolds capable of effectively targeting thioredoxin in vivo. Similarly, the structure of ferroptocide may also provide the basis for the development of new cancer therapeutics. Cancer cells in the mesenchymal state have been shown to be refractory to conventional therapeutic agents and have been linked to metastasis and cancer relapse. Recent studies have illuminated a dependency of this cell state on iron and the vulnerability of these so-called 'persister' cancer cells to ferroptotic cell death[7,8]. It will be interesting in future work to evaluate the ability of ferroptocide and related analogues to eradicate this population of cells in physiologically-relevant cancer settings. Furthermore, persister cancer cells may up-regulate or heavily rely on thioredoxin, which could provide the means to selectively eradicate this population of cells upon small-molecule intervention. Overall, the study from Hergenrother and co-workers may have important implications in cancer biology and translational research. ❐

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