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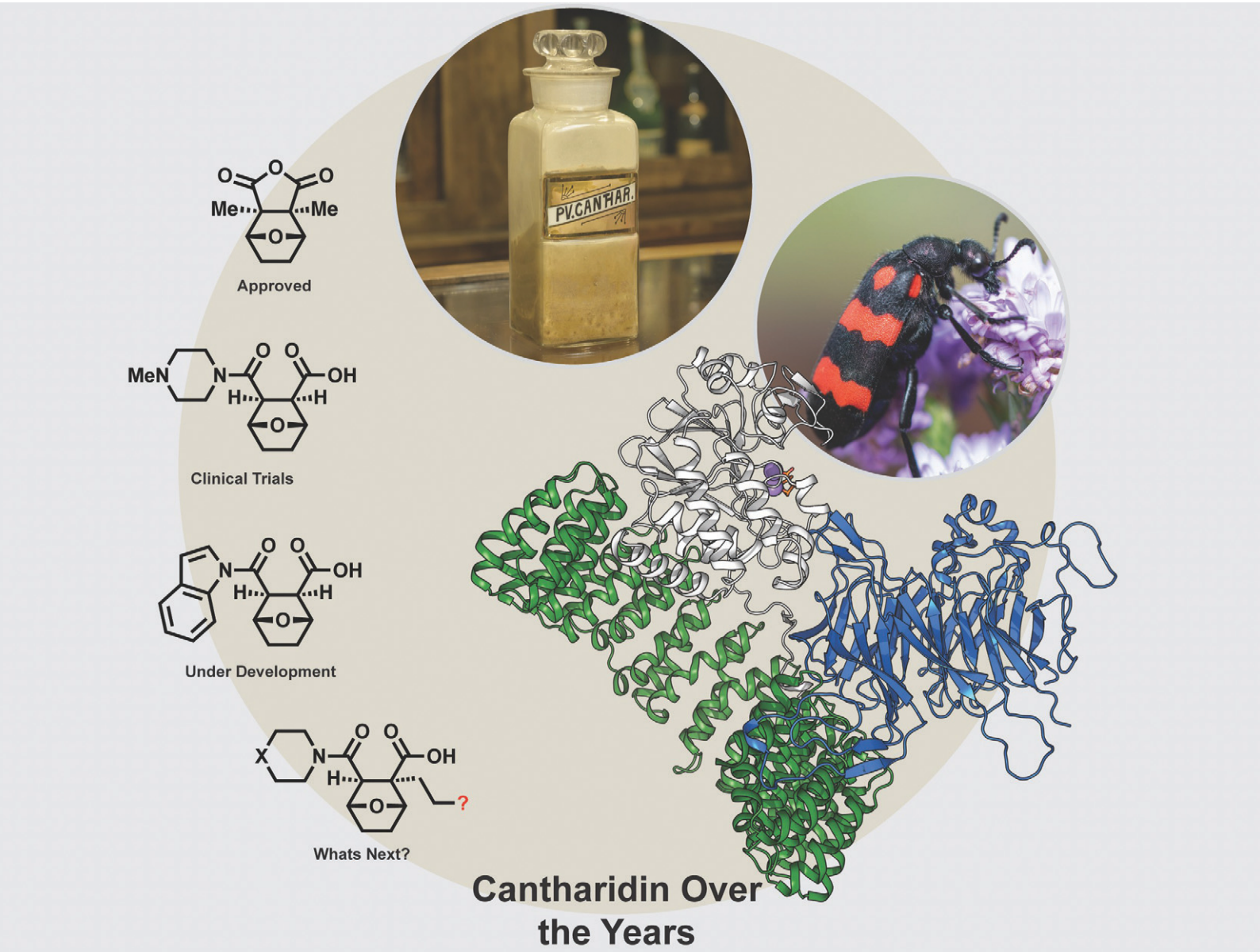

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REVIEW ARTICLE
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On the history, synthesis, and medicinal use of cantharidin, LB-100, and their analogs†

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Cantharidin, a defensive toxin produced by blister beetles, has fascinated chemists, physicians, and historians for centuries. From its notorious use as the aphrodisiac "Spanish fly" to its modern FDA approval as YCANTH™ for molluscum contagiosum, this small yet complex molecule has inspired both infamy and innovation. Over the past hundred years, some of the most eminent synthetic chemists, including Professors Diels, Alder, Ziegler, Schenck, Stork, and Dauben, have tackled the formidable challenges of cantharidin synthesis, establishing benchmarks in organic chemistry. Parallel biological studies revealed cantharidin and its analogs as potent inhibitors of serine/threonine protein phosphatases, particularly PP1, PP2A, and PP5, with wide-ranging implications in oncology, immunology, and chemical biology. Derivatives such as norcantharidin and LB-100 have broadened therapeutic horizons, the latter reaching clinical trials as a novel anticancer agent and immune checkpoint potentiator. Despite inconsistencies in the literature, ranging from pharmacological selectivity to reproducibility of assay data, recent advances in structural biology, computational modeling, and medicinal chemistry have opened new opportunities to refine potency, selectivity, and stability of cantharidin-derived therapeutic molecules. This review critically examines the historical, chemical, and biomedical landscape of cantharidin and its derivatives, clarifies longstanding ambiguities, and highlights future opportunities to develop phosphatase-targeting therapies for cancer, autoimmune, and inflammatory disease.

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Introduction

Foreword

The many aspects of cantharidin's rich history have given rise to numerous literature summaries on focused cantharidin medical,[1] biochemical,[2] or structural aspects[3] of cantharidin, among other topics. Furthermore, meta-analyses[4] have captured bibliometric data including the number and geographic location of studies involving cantharidin. However, to date no one has comprehensively detailed the synthetic challenges associated with synthesizing cantharidin and related structures, efforts toward which spanned most of the 20th century and include a who's who list of eminent researchers. It is now accepted that cantharidin's pharmacological effects arise due to inhibition of protein phosphatases. Synthetic difficulties associated with assembling the parent compound ultimately drove biological studies towards using derivatives of cantharidin's structurally simpler cousin, norcantharidin, which lacks both methyl groups of cantharidin – a feature that facilitates the synthesis but also reduces potency against phosphatases.

The literature surrounding cantharidin and its analogs presents several challenges due to inconsistencies, citation gaps, and evolving interpretations. For instance, while many sources state that cantharidin has been used in traditional Chinese medicine for thousands of years, these claims are often repeated without direct reference to original sources, leading to citation chains that are difficult to verify. Similarly, the cantharidin derivative LB-100 is frequently described as a selective PP2A inhibitor, though direct experimental evidence supporting this specificity is limited, and recent studies suggest a broader target profile. Additionally, reproducibility issues have been noted in studies examining how (nor) cantharidin analogs inhibit serine/threonine phosphatases. Reported IC_{50} values for PP1 and PP2A can vary by orders of magnitude across different studies, with selectivity profiles ranging from PP1-selective to non-selective to PP2A-selective, underscoring the need for standardized assays and careful interpretation of the data.

To gain a comprehensive understanding and overview of the cantharidin field to date, a detailed and critical analysis is essential. In this work, we present such an analysis with

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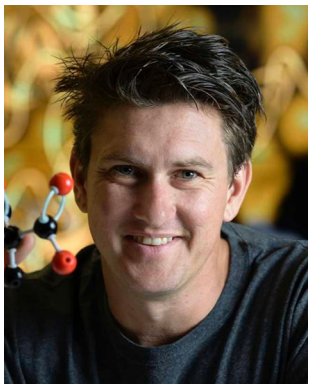
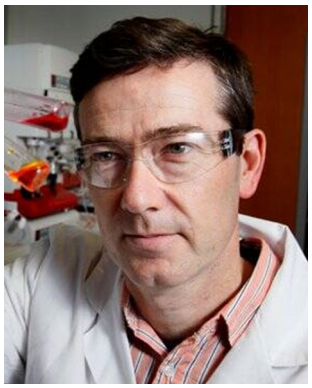
the aim of clarifying longstanding ambiguities, while also highlighting the compound's rich historical context and the remarkable organic chemistry that has guided the development of clinically relevant analogs over the past century. We conclude by looking ahead, considering the renewed clinical interest in cantharidin following its recent FDA approval, the promising oncology outcomes associated with its derivative LB-100, and new biochemical insights into the selectivity of these scaffolds. With emerging evidence suggesting that cantharidin-based compounds may selectively engage a family of six serine/threonine phosphatases implicated in a broad spectrum of diseases, we also explore how recent synthetic innovations may pave the way for therapeutic strategies targeting conditions with significant unmet medical needs.



Kevin A. Scott

Kevin A. Scott – Kevin earned his B.S. in chemistry from the University of California Irvine, where he investigated antifungal potentiators under the mentorship of Professor David L. Van Vranken. In 2015 Kevin began his PhD in pharmaceutical sciences in the College of Pharmacy at the University of Arizona (UofA). Kevin was mentored in his doctoral research by Professor Jon T. Njardarson. During this time Kevin contributed to numerous synthetic methodologies toward substituted phenols, pyridines, and arenes, and developed novel routes toward C1/C2-substituted cantharidin analogs. Following his PhD, Kevin completed postdoctoral work in chemical immunology under Professor Katya Vinogradova at The Rockefeller University, and in evolution and immunology under Professors Koenraad Van Doorslaer, Michael S. Kuhns at the UofA. For the past year Kevin has been an independent OneHealth fellow under the guidance of Professor Wei Wang at the UofA – this fellowship was awarded under the guidance of Profs. Van Doorslaer and Kuhns. Kevin is currently CEO of Teleport Pharmaceuticals and lecturer of medicinal chemistry at the UofA.



Adam McCluskey

Adam McCluskey – Adam is a graduate (BSc(Hons) and PhD) of the University of Strathclyde Scotland. He relocated to the University of Queensland Australia to undertake postdoctoral studies in reactive intermediates chemistry with Prof Curt Wentrup. A change in career direction towards medicinal chemistry with Prof Ronald J Quinn at Griffith University Australia working on the phosphatase inhibitors okadaic acid, microcystin and cantharidin. He has established a thriving medicinal chemistry research team at the University of Newcastle in Newcastle Australia. His teams current focus in the development of small molecule modulators of protein function, most recently targeting the endocytosis proteins clathrin and dynamin GTPase.

Jon T. Njardarson

Jon T. Njardarson – Jon received his Ph.D. at Yale University in 2001 with Professor John L. Wood. Following postdoctoral training with Professor Samuel J. Danishefsky at The Memorial Sloan-Kettering Cancer Center he started his independent career in 2004 at Cornell University. In 2010, Professor Njardarson moved his research group to The University of Arizona.

Wei Wang

Wei Wang – Wei is Coit Professor of Pharmacology and Toxicology and Professor of Chemistry and Biochemistry at the University of Arizona. His research interest includes new organocatalytic and photoredox catalytic synthetic methodology development and medicinal chemistry focusing on targeted protein degradation for cancer and aging. He is also developing bioorthogonal chemistry for protein labeling and functionalization and drug delivery. He has co-authored more than 360 papers, 1 book and filed 12 patents. He is the fellow of The American Institute for Medical and Biological Engineering (AIMBE).

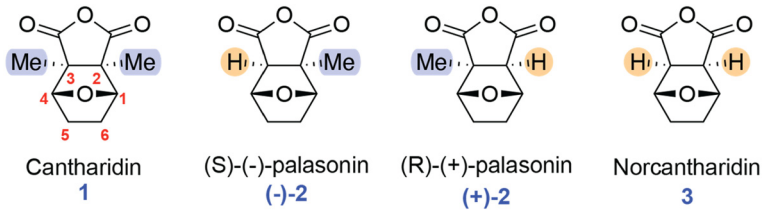


Fig. 1 Structures of cantharidin (left), the related natural product palasonin in both of its enantiomeric forms (center), and the structurally related synthetic compound norcantharidin (right).

History of cantharidin

Meloid beetles, commonly known as blister beetles, produce a potent compound called cantharidin (**1**, Fig. 1) that can cause severe blistering upon contact and is toxic when ingested. Although thousands of species of meloid beetles produce the compound, the species *Mylabris phalerata* and *cichorii* appear in Chinese texts. These texts are the oldest known references to cantharidin in medicinal contexts, dating back over two thousand years; today cantharidin is still used in Chinese folk medicine for its antitumor effects, ability to increase white blood cell count, and for its irritant effects on the urinary system (perhaps not surprisingly, these claims have been recently corroborated by systematic scientific studies described below).[5] Since then, cantharidin has seen fluctuating therapeutic interest across cultures.[6] Historically, it has been used to treat warts, as a purported aphrodisiac, and more recently in folk medicine, where it has been credited with boosting lymphocyte levels (a claim supported by scientific studies) and exhibiting antitumor effects. Modern pharmacology has revealed that cantharidin is a potent inhibitor of the serine/threonine phosphatases PP1, PP2A, PP4, and PP5 – a mechanism that likely underpins both its toxic and therapeutic actions.[7] Some of the notable events surrounding cantharidin and its analogs are highlighted in Fig. 2.

The compound's notoriety extends far beyond its pharmacology. Between the 11th and 18th centuries, cantharidin – often in the form of "Spanish fly" – became infamous in European courts for its alleged aphrodisiac properties. Historical accounts describe its use by figures such as Holy Roman Emperor Henry IV, Roman empress Livia, French sorceress Catherine Monvoisin, and Louis XV (these accounts are published in a 2012 Scientific American article titled "When Sparks Fly: Aphrodisiacs and the Fruit Fly", though these claims cannot be traced to primary sources). Perhaps most infamously, the Marquis de Sade was sentenced to death for secretly administering cantharidin to women in attempts at seduction – an act considered criminal poisoning. Although his death sentence was never carried out, the mythos surrounding cantharidin has endured through centuries of fascination, scandal, and scientific inquiry.[1] Its cultural legacy even made its way into modern entertainment: in the animated series *Futurama*, the episode "Spanish fry" references this legacy when a character has his nose stolen by aliens who traffic in the aphrodisiac "human horn" – a darkly humorous nod to cantharidin's infamous reputation.

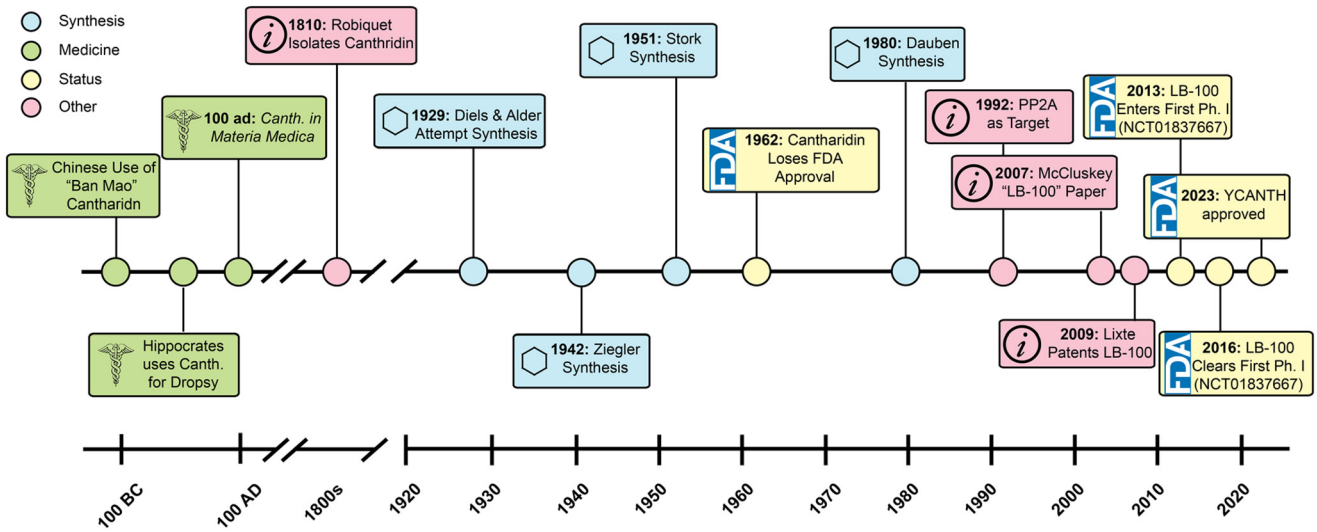


Fig. 2 A timeline of notable events in the history of cantharidin and its derivatives. Synthetic landmarks are shown in blue, landmarks of medicinal use in green, FDA approval status and clinical trials in yellow, with other notable events in pink.

In 1810, French pharmacist Pierre Robiquet became the first to isolate cantharidin – an achievement that stands as one of the earliest known purifications of a pharmaceutically active compound. Robiquet noted that the toxicity of cantharidin, which he considered comparable to that of strychnine, rendered it unsuitable for use as an aphrodisiac.[8] Nevertheless, preparations containing the compound – particularly those marketed as "Spanish fly" – remained popular in the West for precisely that purpose. Despite repeated toxicity warnings, numerous cases of poisoning and several deaths were attributed to its misuse.

Beyond its controversial internal use, cantharidin was widely employed by dermatologists for topical treatment of skin conditions such as warts and molluscum contagiosum. When the Food, Drug, and Cosmetic Act was enacted in 1938, cantharidin formulations were effectively grandfathered in, having met the prevailing safety standards of the time. However, following the 1962 Drug Efficacy Study Implementation (DESI) amendment, the FDA withdrew its approval due to a lack of submitted evidence for efficacy.[1]

Between the 1920s and the 1990s the medicinal value of cantharidin captured the attention of synthetic chemists who took on the challenge of its total synthesis. Several groups completed elegant syntheses all of which required creative solutions for installing the two key methyl groups. Despite comparatively herculean efforts to make this non-selective phosphatase inhibitor more specific and less toxic, researchers achieved relatively little success in generating synthetic analogs. However, a somewhat selective and significantly less potent analog of norcantharidin (Fig. 1), known as LB-100, has been the subject of seven clinical trials for treating cancer and in 2023 cantharidin itself was approved for topical use by the FDA for treating molluscum contagiosum (YCANTH). Herein we will discuss the history, synthesis, and medicinal chemistry of cantharidin and its analogs and the outlook for this amazing molecule.

Biosynthesis, physicochemical properties, and role as a pheromone and allomone

Cantharidin is a defensive toxin produced by over 1500 species of blister beetles. In 2017, Jiang and coworkers published a study in which they knocked down putative genes involved in the biosynthetic pathway of cantharidin to demonstrate the effect of the proteins in this pathway on downstream metabolites, showing that three specific genes encode enzymes (methyl farnesoate epoxidase, juvenile hormone acid *O*-methyl transferase, and juvenile hormone epoxide hydrolase) that transform methyl farnesoate into juvenile hormone III diol (Fig. 3a) which serves as a precursor to cantharidin.[9]

Cantharidin is a chameleonic molecule, having different physicochemical properties depending on cellular context.

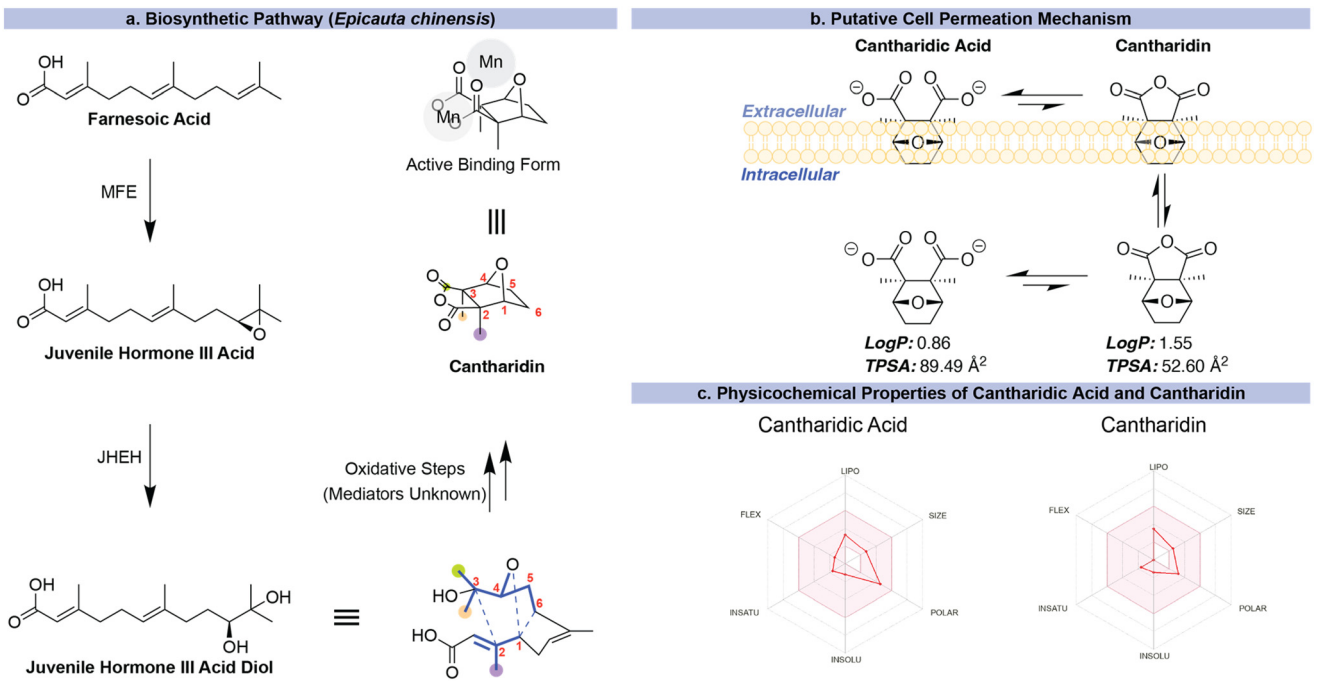


Fig. 3 Biosynthesis and physicochemical properties of cantharidin. a. Cantharidin has evolved a compact structure that is biosynthetically generated from juvenile hormone III diol, which is produced from farnesoic acid by an enzyme cascade including methyl farnesoate epoxidase (MFE) and juvenile hormone epoxide hydrolase (JHEH) – the transition state and atom mapping in this sequence are speculative; b. the molecule has evolved to be able to cross the cell membrane despite having high negative charge density, possibly due to its equilibrium between the diacid and the anhydride; c. both cantharidin and its active form, cantharidin acid, have properties that are considered to be both drug-like and developable (clockwise from top: LIPO = lipophilicity, SIZE = size (mw, g mol^{-1}), POLAR = polarity, INSOL = insolubility, INSATU = insaturation, FLEX = flexibility).

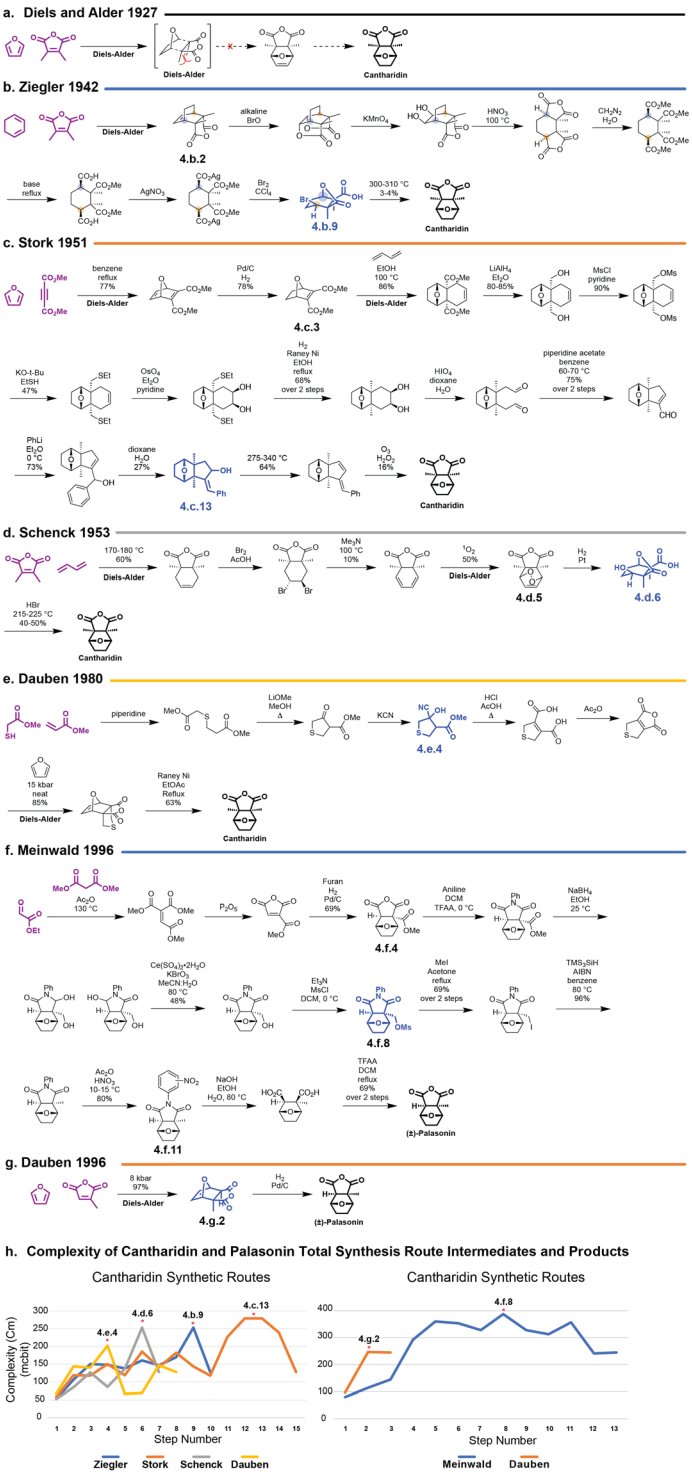


Fig. 4 Total syntheses of cantharidin and Palasonin. a. Otto Diels and Kurt Alder attempted a two-step synthesis in 1927 but the product failed to undergo the pericyclic reaction; b. Ziegler (under the guidance of Schenck) carried out the first total synthesis of cantharidin in 1942 in 11 linear steps; c. Gilbert Stork forged another total synthesis beginning with a different Diels–Alder reaction, culminating in a 14-step route in 1951; d. in 1953, Schenck optimized the total synthesis of cantharidin, publishing a clever 7-step route utilizing a singlet oxygen Diels–Alder reaction; e. although Dauben's 1980 synthesis of cantharidin was one more step than Schenck's, the yield was considerably higher; f. Rydberg and Meinwald carried out a lengthy synthesis of cantharidin's cousin, palasonin, in which 100% of the heavy atoms were in place after three steps, though an additional nine steps of redox manipulation and protection/deprotection to change the methyl ester to a methyl group; h. the complexity landscapes of all the syntheses shown here are depicted for easy comparison of route efficiency, with cantharidin syntheses on the left and palasonin syntheses on the right.

One may assume that under physiological conditions, the reactive anhydride of cantharidin would open to the more stable diacid, cantharidic acid (Fig. 3b). The calculated log*P* and tPSA of cantharidin are 1.55 and 52.60 Å^2, respectively, while those of cantharidic acid are 0.86 and 89.49 Å^2 (calculated using the SwissADME webserver).[10] While one would not expect cantharidic acid, with its high negative charge density, to passively diffuse across membranes.[11] However, an equilibrium driven diffusion beginning with the hydrophobic end of cantharidin presents a plausible mechanism for cell entry (Fig. 3b). Many phosphatase inhibitors that bind to the active site have negative charge density similar to cantharidin, mimicking the charge of the phosphate group, which would typically bind there (see Fig. 13 for examples of natural product phosphatase inhibitors). This may be an intriguing mechanism to extend to other phosphatase inhibitors. Overall, SwissADME predicts the physicochemical properties of both cantharidic acid and cantharidin to be well within the range considered to describe "drug-like" molecules[12] (Fig. 3c).

Cantharidin's infamous reputation as an aphrodisiac extends into the insect world. In some beetle species that do not produce the compound, males actively seek out and consume it, and then offer it to females as a nuptial gift.[13] The females, in turn, use the toxin to protect their eggs – a remarkable example of ecological co-option. This behavior underscores cantharidin's value in the field of chemical ecology, a discipline born from the intersection of entomology and synthetic organic chemistry. By synthesizing insect hormones and allomones, chemists have been able to explore their roles in insect communication and social behavior. These investigations helped drive interest in the cantharidin scaffold and related molecules such as palasonin (**2**, Fig. 1) and norcantharidin (**3**, Fig. 1) derivatives.

Chemistry

Synthesis of cantharidin

Here we present a stepwise representation of the published total syntheses of cantharidin (Fig. 4a–e) and palasonin (Fig. 4f and g). To better understand the challenges associated with these syntheses, we quantified and visualized the efficiency of each synthesis using a graphical representation[14] of the molecular complexity[15–17] at each step of the synthetic route. In this way, we create a landscape of complexity which resembles a mountain range (Fig. 4h). The analogy of the synthetic route, then, is that of a mountain climber: an optimally concise synthesis is represented by directly scaling the face of the mountain, while a complex synthesis involving many manipulations is analogous to a wanderer meandering over multiple peaks before reaching the summit.

Each cantharidin synthesis involved an intermediate with far greater complexity than the final product (highlighted with an asterisk, and the compound in blue in each corresponding synthetic route), illustrating the difficulty of navigating the architecture of this information-dense molecule. The inefficiency inherent in these "complexity peaks" are reminiscent of the drop in complexity imparted by protecting group manipulations described in Professor Baran's definition of an "ideal synthesis".[18] We will look at each synthesis in detail. Each of these syntheses can also be viewed sequentially on Professor Njardarson's application (app) Chemistry By Design, with additional features such as the "quiz" mode.[19]

Cantharidin is structurally simple, yet constrained. It is a symmetrical monoterpene derived from two isoprene units. The synthetic approaches to this scaffold are deeply rooted in the German chemistry literature, beginning in 1927, when Professors Diels and Alder submitted a paper in which they postulated that they could synthesize the core scaffold in a single step from dimethyl maleic anhydride and furan using the pericyclic reaction that bears their namesake.[20] Indeed, in a 1928 paper, Professor Franz von Bruchhausen demonstrated that a thermal catalytic decomposition of cantharidin vapor by passing it over palladium on asbestos at ~280 °C gives dimethyl maleic anhydride and furan, "if not quantitatively, then very cleanly".[21] This retrosynthesis gave validity to Diels and Alder's proposed route. Alas, due to the steric hindrance experienced by the methyl groups that would end up only 2.7 Å apart, the reaction did not come to fruition (Fig. 4a).[22] Other groups would go on to carry out successful syntheses of cantharidin, but in nearly each case, the redox manipulations required to install both the vicinal methyl groups required several steps.

Ziegler's 1942 synthesis

After Diels and Alder's attempt at synthesizing cantharidin, Professor Ziegler published a remarkably detailed 79-page treatise on the synthesis of cantharidin (*Die Synthese des Cantaradins*).[23] Ziegler noted that the methyl groups which preclude a Diels–Alder cycloaddition with furan (Fig. 4a) do not preclude the analogous cycloaddition with cyclohexadiene. This reaction gives the less strained [2.2.1]-fused ring system of compound **4.b.2**, which, although it only barely resembles the final product, could be manipulated in a series of redox steps to achieve the first total synthesis of cantharidin in 1942 in eleven linear steps (Fig. 4b).[23] The penultimate intermediate **4.b.9** has the highest complexity score. Although the final step gave only 3–4% of cantharidin, the impressive scope and detail of this work enumerated ~50 compounds and included detailed drawings of glassware setups for keeping sensitive reactions under dry and inert conditions.

Stork's 1951 synthesis

In 1951 Professor Gilbert Stork improved upon Ziegler's pioneering work with an increase in overall yield. However, despite over 90% of the atoms being in place after the first step, the additional 12 steps required to realize complete assembly of cantharidin left much room for improvement

(Fig. 4c).[24,25] The initial Diels–Alder cycloaddition and selective reduction of the distal olefin gave the excellent dienophile **4.c.3**. After a second Diels–Alder cycloaddition, the insipient methyl groups existed as geminal bis-methyl esters in the endo position, with the resulting cyclohexene ready to be oxidized to set up for a strategically inefficient series of manipulations to form the anhydride. To finish the synthesis, the complex benzylidenecyclopentenol derivative **4.c.12** was first dehydrated by pyrolysis and then ozonolyzed before decomposing the ozonolide with hydrogen peroxide to give cantharidin in 16% yield.

Schenck's 1953 synthesis

Professor Günther O. Schenck, a pioneer of photochemistry, published the shortest synthetic route to cantharidin (7 steps) by utilizing a clever singlet oxygen Diels–Alder reaction to install the bridgehead oxygen in 1953 (Fig. 2d).[26] Schenck noted that the endoperoxide **4.d.5** possessed the same spatial orientation as cantharidin (with respect to the methyl groups), and that its decomposition presented a route to the target compound. Lactone **4.d.6**, the most complex molecule of this route, was decomposed under acidic conditions, *via* an internal S_N2 to give cantharidin.

Dauben's 1980 synthesis

In 1980, Professor William Dauben and coworkers showed off the high-pressure Diels–Alder chemistry that had been developed in his laboratory by tackling cantharidin. The team assembled dienophile **4.e.4** in 5 steps, which essentially comprised a version of dimethyl maleic anhydride in which the methyl groups were restrained by a bridging sulfur atom. This minimized the degrees of freedom, effectively reducing the proximal size of the dienophile sufficiently to enable the Diels–Alder reaction to proceed (Fig. 4e).[27] RANEY® nickel reduction of the thioether gave cantharidin in 63% yield.

Historically, medicinal use of cantharidin has been facilitated by direct purification from insects, and at the time of this writing, scores of patents in the US and worldwide describe methods for purifying the compound from natural sources. It is remarkable that after a burst of interest spanning 50 years, no total synthesis of cantharidin has been published, marking a lull in the field of nearly the same span of time. Currently, the commercial production of cantharidin for topical use by Verrica Pharmaceuticals is highly similar to the Dauben route.[28]

Synthesis of palasonin

Cantharidin has a structurally simpler natural product relative, palasonin ((±)-**2**, Fig. 1), which contains only one methyl group instead of the two found in cantharidin. This compound was first isolated from *Butea frondosa*, a tree long used in traditional Indian medicine.[29] As previously noted, cantharidin is consumed by some beetles, and is known to be a chemical attractant of beetle species that don't produce the compound. In 1996 Professor Jerrold Meinwald and coworkers – pioneers in the emerging field of chemical ecology – undertook the synthesis of palasonin to investigate whether it elicited behavioral responses in beetles similarly to its dimethyl counterpart (cantharidin).[30] However, after completing the chemical synthesis the group did not publish a follow-up study on the role of palasonin as an allomone in beetles. One study reported that at least two species of *Hycleus* beetle produce palasonin,[31] though at levels 12–20 times lower than the amount of the cantharidin they produce – suggesting that perhaps a semiochemical role for the compound remains to be discovered.

The first chemical synthesis of palasonin turned out to be more challenging than expected. Palasonin lacks one of the two methyl groups that made the cantharidin synthesis so difficult; despite this, 100% of heavy atoms were in place in compound **4.f.4** after step 3 yet the remainder of the synthesis required an additional 11 steps of protection/deprotection and redox manipulations (Fig. 4f). Eight of the protection and redox steps took place in complexity space higher than that of the product, indicating a challenging manipulation of reactive moieties, with **4.f.11** having the highest complexity score. Intermediate **4.f.13** was generated by nitration of the phenyl protecting group, which could then be hydrolyzed before anhydride ring closure.

The last paragraph of Rydberg and Meinwald's palasonin synthesis pays compliment to the "elegant syntheses of cantharidin" by Professors Stork and Dauben, calling them "achievements of great esthetic and intellectual appeal". Professors Rydberg and Meinwald go on with humility, stating that their own synthesis of palasonin does not embody insight or imagination comparable with those of Stork and Dauben, but that, as chemical ecologists, their synthesis opens the door to studying the role of these chemical messengers in the lives of insects.

When Dauben followed up with his own synthesis the just a few months later, he took a rather different tone, noting that "Rydberg and Meinwald reported a total synthesis [giving] crude [palasonin] in poor yield…" and added pointedly that "the crude material was purified by [inconvenient] preparative gas chromatography".[32] Dauben's synthesis, though, was remarkably elegant, highlighting once again the utility of the high pressure Diels–Alder reaction that was showcased in the cantharidin synthesis from the same group. Professor Dauben and coworkers wrapped up palasonin in an impressive two steps from furan and methylmaleic anhydride (Fig. 4g), mirroring what Professors Diels and Alder had hoped to achieve with cantharidin nearly 70 years earlier. Efforts to directly produce cantharidin with the same high-pressure chemistry were unsuccessful.

Derivatives

C2 and C3 substituted derivatives

Cantharidin (**1**) features methyl groups at the C2 and C3 positions, whereas palasonin has a single methyl group and one proton ((±)-**2**, Fig. 1). Norcantharidin (**3**, Fig. 1), on the

other hand, has no methyl groups, but two protons at these positions. The methyl groups are thought to preorganize and conformationally restrict the diacids of cantharidin and palasonin, thereby enhancing PP2A inhibitory activity relative to norcantharidin. The increased conformational constraint – which is more pronounced in cantharidin than it is in palasonin – may account for their difference in potency.[2,33]

In the previous section we discussed Professor Dauben's remarkably concise high-pressure Diels–Alder approaches to cantharidin and palasonin, in which the C2/C3 substitutions were pre-installed on the anhydride. In 1999, Professor Laidley and coworkers published a follow-up paper investigating cantharidin analogs, which was dedicated in memory of Professor Dauben, and in which he was honored posthumously as an author. This study uses the cantharidic acid (anhydride opened cantharidin) radioligand ([^{3}H]CA) binding assay[34] to measure binding affinity of C2 and/or C3-substituted cantharidin analogs.[35]

The high-pressure chemistry developed by Professor Dauben for the synthesis of cantharidin and palasonin provided an inroad to other singly modified C2 or C3 analogs (Fig. 5a) and doubly C2/C3 modified analogs, in which the endo-facing methyl groups are fused in a 5-membered carbocycle or sulfur heterocycle (Fig. 5b). In addition to the new structures, the authors included in this study a dose response for all new compounds using a competition assay for ^{3}H-cantharidin, giving crucial insights into the importance of the C2/C3 substitutions, which offer varying degrees of conformational constraint to the overall structure. For the first time, a synthetic cantharidin analog with a greater affinity for PP2A than cantharidin itself was discovered in compound **10**.

Norcantharidin analogs

The main synthetic challenge that we covered in the total synthesis of cantharidin and palasonin was the installation of the C2/C3 methyl groups. Laidley's use of Dauben's high-pressure Diels–Alder chemistry provided valuable insights into structure–activity relationships (SAR) at these positions (Fig. 5b). However, large-scale exploration of analogs with selective PP2A activity ultimately relied on simpler strategies, such as ring-opening of norcantharidin with nucleophiles (*e.g.*, amines) or modification at the C1/C4 and C5/C6 positions *via* substituted furan precursors for the Diels–Alder reaction, which became the dominant focus of the following decade.

In 1996 and in 2000, one of us (AM) published a pair of papers outlining cantharidin-derivative SAR for PP2A inhibition that set the stage for the coming decade of analogs (Fig. 6).[36,37] By removing the bridgehead oxygen and replacing it with a methylene group, it was determined that the oxygen is required for binding (Fig. 6a), which is consistent with Tatlock's 1997 computational model of cantharidin binding to PP2B and D'Arcy's 2019 crystal structure of LB-100 binding to PP5 (*vide infra*),[38,39] in which the bridgehead oxygen coordinates with the metals at the active site of the phosphatase. Removal of the methyl groups in positions C2 and C3 reduces activity significantly, and addition of groups to C1/C4 or C5/C6 completely inhibits PP2A activity. This is interesting because Sodeoka notes that removal of substituents at C2/C3 increases PP2B inhibitory activity, and substituents at C1/C4 and C5/C6 is tolerated by PP2B inhibitors, suggesting that there may be a key to phosphatase selectivity here.[40] In addition, anhydride ring

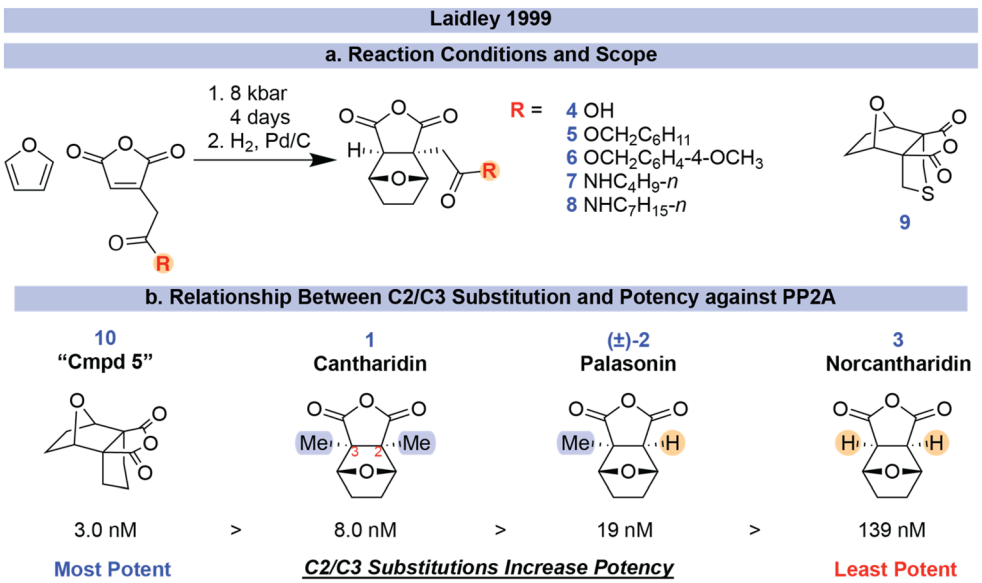


Fig. 5 The first C2 and C3 substituted cantharidin analogs and relative potencies against PP2A. a; High pressure Diels–Alder chemistry to synthesize the cantharidin core was used to generate a variety of C2- and C2/C3-substituted cantharidin derivatives; b; subsequent competition assays using tritiated cantharidin identified a structural relationship between C2 and C3 substitution and binding affinity for PP2A, in which the relationship was determined to be that the greater the substitution the greater the potency.

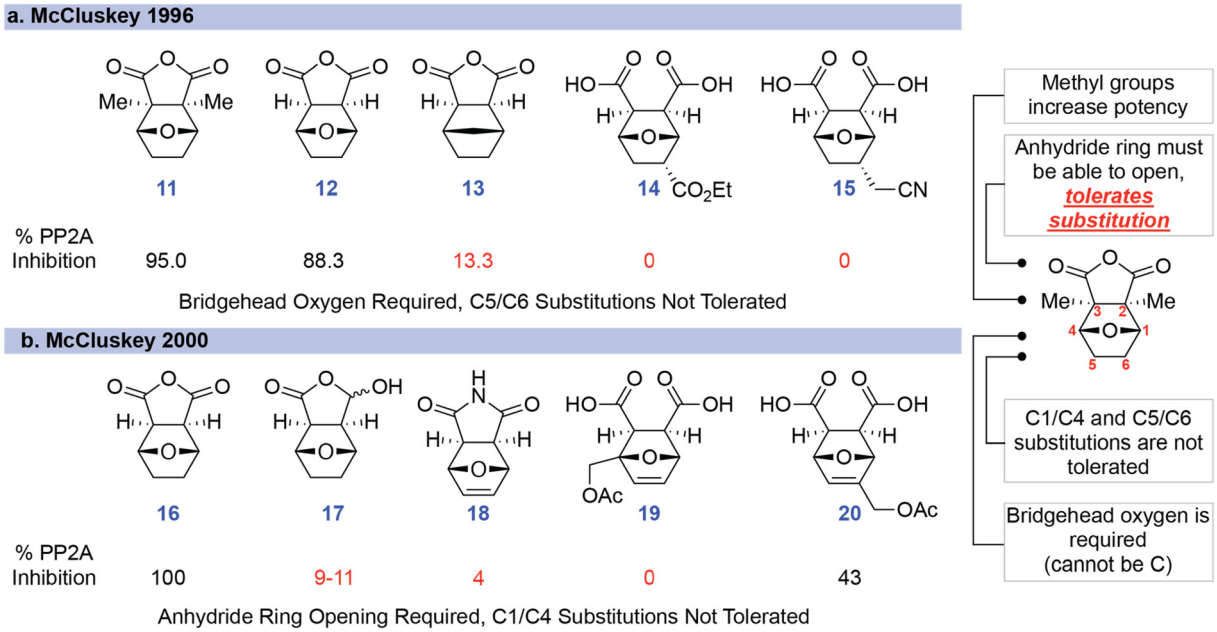


Fig. 6 A functional understanding of the structure/activity relationships between substituted norcantharidins and PP2A. Percentage PP2A inhibition measure at 1 mM compound concentration.[36]

opening was also determined to be a requirement for binding, because the resulting diacids also coordinate with the active site metals, and the negative charges are reminiscent of those of a phosphate group which would typically occupy this space (Fig. 6a and b). The understanding at this time was that the methyl groups increase potency, the anhydride ring must be able to open but tolerates substitution, C1/C4 and C5/C6 substitutions are generally not tolerated, and the bridgehead oxygen is required for binding. Reduction to the anhydride to a lactone, and conversion to the imide both reduced the activity to near zero, as did substitution at C1/C4 (Fig. 6b)

A flurry of papers from the University of Newcastle group (Newcastle group; Adam McCluskey, Jennette Sakoff and coworkers) in the early 2000's was a tour de force of norcantharidin analogs as PP1 and PP2A inhibitors.[36,41–46] These studies share a rigorous attention to detail with appropriate controls in each study and in particular, the reporting of negative results, which can be incredibly useful. The importance of attention to detail is emphasized by the range of values obtained by the internal controls within these studies (namely using cantharidin and norcantharidin as PP1 and PP2A inhibitors as a baseline) which are highlighted in blue in Fig. 7. The IC_{50} values span more than a 20-fold range (as in cantharidin inhibiting PP1) and range from 10-fold selective for PP2A (cantharidin, Fig. 7a),[47] to non-selective (norcantharidin, Fig. 7c),[46] to nearly 2-fold selective for PP1 (cantharidin and norcantharidin, Fig. 8a).[42]

A 2000 paper from the same group investigated the activity of mono-esters of norcantharidin and found that small esters were tolerated, maintaining sub-micromolar activity against PP2A (**21**, **22**, and **23**, Fig. 7a).[47] These levels were on the same order of magnitude as those for cantharidin, and nearly ten-fold better than norcantharidin. Activities against PP1 remained on the same order of magnitude as both cantharidin and norcantharidin (low micromolar).

In 2001 a study revisited the professed requirement for anhydride ring opening to the dicarboxylate with evidence that the equivalent mono-acids, mono-esters maintained PP1 and PP2A inhibition. Extension of this idea resulted in the synthesis of the norcantharimides, of which the D/L-histidine analogues **25** and **26** showed excellent phosphatase inhibition (Fig. 7b).[41] Interestingly, early molecular docking studies suggested two potential active site binding modes: head-first or feet-first, that rationalized the lack of D/L stereochemical preference. In so doing, this study suggested that modifications to the anhydride moiety that installed and acidic and basic component were tolerated.

A 2002 study reporting the first cantharidin analogs selective for PP1 over PP2 gave some results that were surprising for a number of reasons.[46] First, the norcantharidin internal control did not indicate much (if any) selectivity for PP2 over PP1 (Fig. 7c). Furthermore, switching from an oxygen bridgehead to sulfur ablates activity (as in the change from **27** to compounds **28** and **29**). However, this lack of activity suddenly reversed with the maleimide which also has the sulfur bridgehead, but also cannot undergo ring-opening (Fig. 7c), which was previously determined to be a prerequisite for PP2A binding. The conclusion of this study seems to be that the rules of engagement for PP2A don't seem to be entirely the same as for PP1, although this is not directly addressed by the authors.

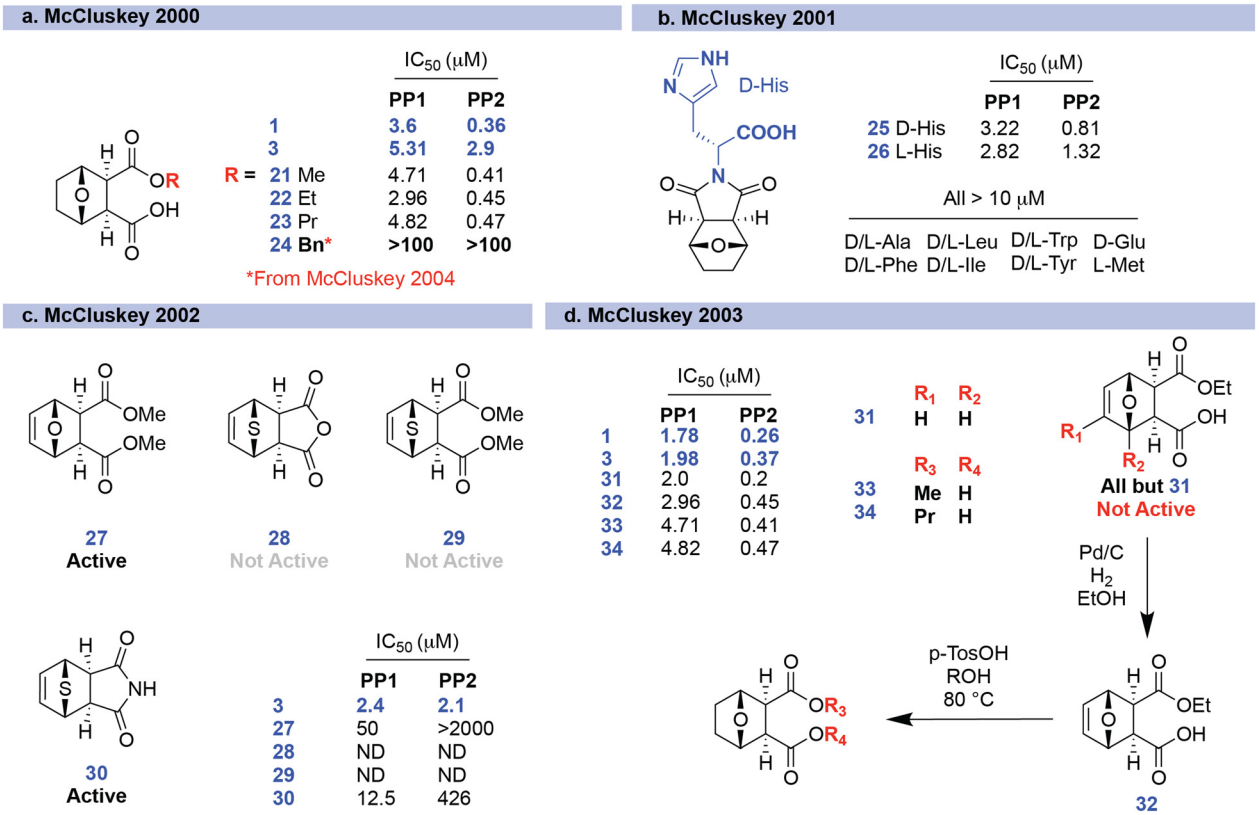


Fig. 7 McCluskey publications from 2000–2003: further SAR studies focused on norcantharidin diacid, acid/amide, and imide moieties. a. Some mono esters maintained relatively potent activity; b. both D- and L-amino acid-derived imides maintained activity but all other tested amino acids had dramatically reduced activity; one diester with an oxygen bridgehead and the unreduced double bond from the Diels–Alder reaction, and an unsubstituted imide with a sulfur bridgehead and the same unreduced double bond exhibited modest activity, but great selectivity for PP1 over PP2A; one monoester with unreduced double bond and two monoester norcantharidin derivatives exhibited decent activity for both PP1 and PP2A, with ~10 fold selectivity for the latter.

In 2003, the Newcastle group explored the impact on PP1, PP2A and cytotoxicity of a small library of mono- and diester norcantharidin analogues (Fig. 7d) and the encapsulation of one of the carboxylate moieties as the lactone in (3a*S*,6*R*,7a*R*)-1-oxohexahydro-3*H*-3a,6-epoxyisobenzofuran-7-carboxylic acid[48] (Fig. 15d, 2003). In this study, phosphatase activity was lost with scaffold substituents and with diester analogues. Good activity was noted in all mono-ester cases: The mono-methyl ester of norcantharidin, with both the un-reduced C5–C6 ethylene bridge (**31**) and the fully reduced (**32**) both maintained good activity against PP1 and PP2A (Fig. 7d). Despite no phosphatase activity, the diester analogues retained good broad-spectrum cytotoxicity against a panel of cancer cell lines. Presumably a consequence of cellular esterase activity yielding endothall. As has been the case in all development of mono-ester and mono-amide norcantharidin analogue synthesis no effort was made to control the stereochemical outcome of the resultant additions. Given it is expected the cantharidin analogue geometry affects potency, the phenyl/ethyl diester analogue separated (by chromatography) into four diastereomeric pairs. This analogue is phosphatase inactive, but each diastereomeric pair returned essentially identical cytotoxicity outcomes.

A 2004 paper from the same group investigated the potency of mono-amides containing the unreduced C5–C6 ethylene bridge and discovered that aryl amides like **33** and dimethyl amide of norcantharidin **34** maintained decent phosphatase inhibitory activity and slight selectivity for PP2A over PP1 (Fig. 8a).[42]

In 2007, the Newcastle group published a study in which more than a dozen analogs were tested *in vitro* against PP1 and PP2A and screened against 9 different cell lines.[49] Researchers often exclude compounds that lack data if, for example, they are insoluble. In Table 2 of the previously published paper (compound **37**; corresponding to Fig. 8b in this article), the authors noted that compound **37** was insoluble in DMSO, and as a result, no activity values were reported—an omission that would unexpectedly shape subsequent oncology research. In the two years following this publication, two companies, Genetic Technologies and Lixte Biotech Holdings (hereafter Lixte), patented this compound and Lixte took it to clinical trials in the US under the name LB-100. It is probable that this analogue was developed independently by the Lixte team at the same time as by the Newcastle team.

A 2008 study by the Newcastle Group tested many alkyl amides of norcantharidin (Fig. 8c). Even the best ones, **38**

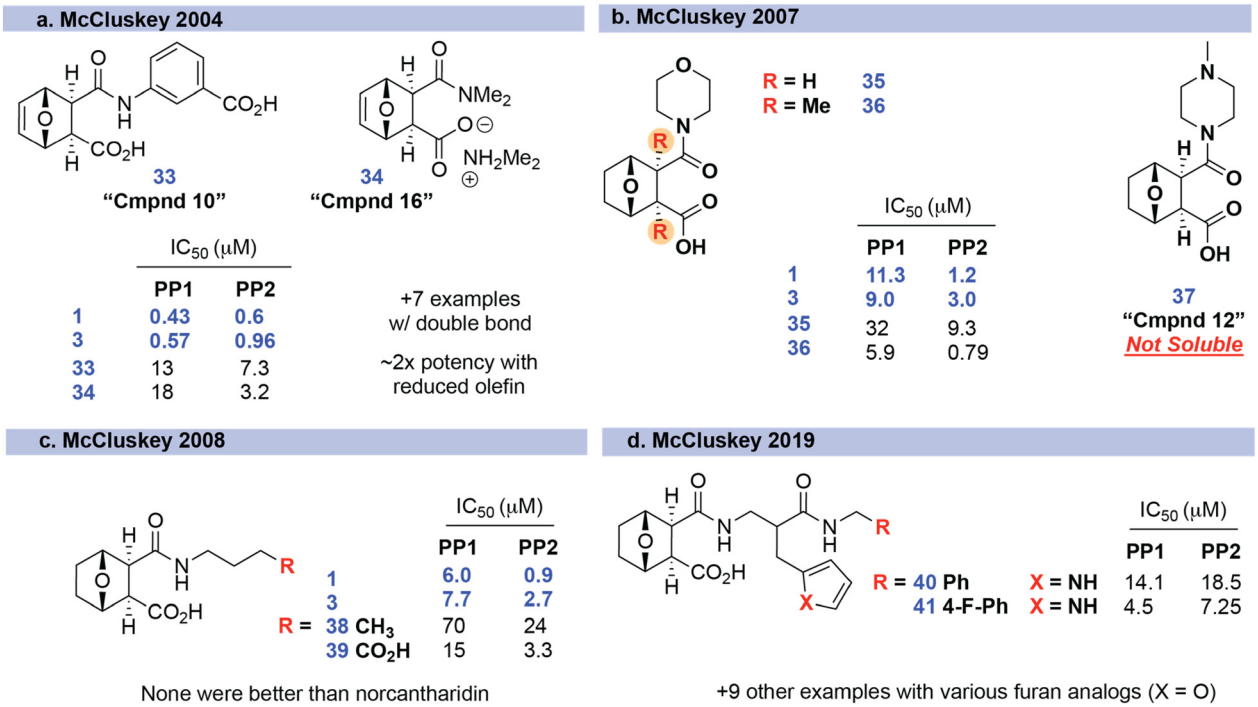


Fig. 8 McCluskey publications from 2004–2019. a. Some substituted amides shoed decent potency for both PP1 and PP2A, with ~2 fold selectivity for PP2A; b. various cyclic amide derivatives of norcantharidin were tested and shown to have good activity against PP2A, with the cantharidin derivatives being most potent – compound 12 would end up being renamed LB-100 and taken to the clinic by Lixte Biotechnologies; c. various open-chain amides were tested and some were shown to have low micromolar activity against PP2A, though none were as potent as norcantharidin; d. elaborated amides with greater complexity than those in panel "c" were generated and tested, with a 4-F-Ph derivative showing greatest potency with PP1 and PP2 inhibitor effects on the same order of magnitude in the mid micromolar range.

and **39**, were not better than cantharidin or norcantharidin against either PP1 or PP2A.[44] Once again, the thoroughness of the group commendably published data that are valuable to the field in expanding our understanding of (nor) cantharidin SAR with regard to PP1 and PP2A.

In 2019, Hizartzidis and McCluskey published another interesting series of compounds with PP2A inhibitory activity (Fig. 8d).[50] In efforts to modify the bridgehead position (C1/C4) similar to Baba's independent reports, modified furans were subject to norcantharidin synthesis conditions. This afforded a family of octahydroepoxyisoindoles, which, in keeping with earlier reports, removed all phosphatase activity (shown in Fig. 15d). Additionally-modified furan and pyrrole analogues to preassembled norcantharidin gave rise of a series of novel norcantharidin-amide hybrids. Within this series, the introduction of a phenyl moiety (Fig. 8d) gave modest phosphatase activity, with the 4-fluoro-phenyl analogue 4-fold PP1 and 3-fold PP2A active than the phenyl substituted analogue. This suggests potential additional scope for phosphatase potency enhancements through modification of this moiety.

LB-100

LB-100 has been cited nearly 100 times since its development and patenting by Lixte (the same compound was pursued, but later abandoned, by the Newcastle group). The scope of its reported biological activities is remarkable. Although LB-100 is frequently characterized as a PP2A inhibitor, it remains uncertain whether its antitumor effects derive primarily from the inhibition of a single phosphatase or from a broader, multi-faceted pharmacology; the prevailing evidence supports the latter.[7] Consistent with this view, LB-100 demonstrates activity within several pathways and across a wide range of disease models (Fig. 10), suggesting a mechanism of action that is inherently pleiotropic. Furthermore, potential effects downstream of PP5 introduce a polypharmacological aspect to the equation. Several reviews have examined PP2A as a therapeutic target in oncology and outlined some of LB-100's molecular mechanisms in detail.[7,51,52] We expand on those insights in the following sections.

A 2018 study focusing on triple negative breast cancer showed that LB-100/cisplatin cotreatment led to a greater reduction in tumor volume than treatment with cisplatin alone in a TRAIL-resistant MDA468 TNBC mouse model.[53] The enhanced effect was attributed to increased apoptotic cell death, as evidenced by cleaved PARP, which was absent in singe-agent treatments. Importantly, LB-100 displayed antitumor activity both as monotherapy and in combination, without causing significant toxicity or weight loss in mice.

LB-100 treatment in combination with radiation therapy led to prolonged survival of xenograft mice compared with radiation therapy by itself.[54] This is because PP2A inhibition

by LB-100 increased double-strand breaks in DNA, inducing mitotic catastrophe and cell death in irradiated meningioma cells. One of us (AM) has previously demonstrated that norcantharidin analogs accelerate the cell cycle by essentially abrogating G2,[55] suggesting a plausible mechanism for the apparent enhancement of irradiation by LB-100.

Tumors often take advantage of negative regulators of immune function, like programmed death-1 (PD1) and cytotoxic T lymphocyte-associate protein 4 (CTLA-4) to evade immunosurveillance and create a general immunosuppressive environment.[56] In a landmark 2018 preclinical study, Ho and coworkers (along with John Kovach from Lixte) showed that PP2A inhibition by LB-100 can profoundly remodel the tumor immune microenvironment and enhance the efficacy of anti-PD1 checkpoint blockade. The team demonstrated that LB-100 exhibited synergistic effects with anti-PD-1 and elicited a durable antitumor response mediated by T cells in a murine CT26 colon cancer model, a murine carcinoma in which PD-L1 is highly expressed but which responds poorly to anti-PD-1 therapy.[57] Moreover, the investigators demonstrated that LB-100 turns a cold tumor hot by decreasing the number of Tregs in the tumor and increasing CD8+ cytotoxic T cell infiltration. These results corroborate earlier observations that okadaic acid, a potent natural product PP2A inhibitor, activates T cells by modulating membrane signal transductions at low concentrations; notably, it has the opposite effect at high concentrations.[58] In addition to directly activating T cells, okadaic acid is known to abrogate CTLA-4-mediated suppression of T cell activation by inhibiting Akt phosphorylation.[59]

Maggio and Ho, together with Zhuang and Lixte, demonstrated in a similar study focusing on LB-100/PD1 blockade that LB-100 together with anti-PD-1 and 25% of mice receiving combination therapy had complete tumor regression.[60] LB-100 was also shown to increase expression of PD-L1 on tumor cells by enhancing IFNg secretion by CD8+ T cells. Furthermore, the team showed that combination therapy increased CD8+ tumor infiltration and activation, with 50% of tumor-isolated T cells being CD45+ compared with 34% in the control, 32% in LB-100 treated, and 37% in anti-PD-1 treated cohorts.

This foundational preclinical research demonstrated that inhibiting PP2A with LB-100 induces immunogenic cell death and enhances the efficacy of PD-1 checkpoint blockade in murine tumor models. This work provided a compelling rationale for combining LB-100 with immune checkpoint inhibitors to potentiate antitumor immunity. Building on these findings, a phase 1b clinical trial (NCT06012734, entry 6, Table 1) has been initiated to evaluate the combination of LB-100 and atezolizumab (a PD-L1 inhibitor) in patients with microsatellite stable metastatic colorectal cancer – a population typically unresponsive to immunotherapy. The trial is sponsored by the Netherlands Cancer Institute (NKI), with LIXTE Biotechnology supplying LB-100 and F. Hoffmann-La Roche (Roche) providing atezolizumab and financial support through the imCORE Network, a global academic-industry partnership aimed at accelerating cancer immunotherapy research.

Roche's clinical success with atezolizumab, a monoclonal antibody targeting PD-L1, was developed by its subsidiary Genetech and approved as a second-line therapy for urothelial carcinomas while it was being further developed for hematological malignancies and solid tumors.[61] Given the previous observation that LB-100 potentiates the efficacy of a-PD-1 antibodies in mice, and that PD-1 is the native ligand for PD-L1, it stood to reason that LB-100 may also potentiate a-PD-L1 therapies as well. A 2021 study investigating this idea in small cell lung cancer (SCLC) confirmed this hypothesis, demonstrating that LB-100 enhanced the antitumor activity of Genentech's atezolizumab, leading to significant tumor regression and prolonged survival in preclinical models. These results highlighted the potential of PP2A inhibition as a combinatorial strategy to overcome immune resistance and

Table 1 Clinical trials involving LB-100. The first two clinical trials involving LB-100 have completed, which the remainder are either recruiting or active and no longer recruiting. The most recent trials involve combinations with Roche's anti-PD-L1 antibody atezolizumab. The range of interventions with which LB-100 is being combined spans a great portion of the oncological therapy repertoire

Trial number	Date	Phase	Combination	Sponsor	Cancer	Enrollment	Status
NCT01837667	Feb 2013	Ph.I	Docetaxel	Lixte Biotechnology Holdings, Inc.	Solid tumors	29	Completed
NCT03027388	Jan 2019	Ph.II	Brain surgery	National Cancer Institute	Recurrent glioblastoma	7	Completed
NCT03886662	May 2019	Ph. I/II	None	Lixte Biotechnology Holdings, Inc.	Low or Intermediate-1 risk myelodysplastic syndromes	47	Recruiting
NCT05809830	May 2023	Ph. I/II	Doxorubicin	Grupo Espanol de Investigacion en Sarcomas	Advanced soft tissue sarcomas	152	Recruiting
NCT06065462	Aug 2024	Ph. I/II	Dostarlimab	MD Anderson Cancer Center	Ovarian clear cell carcinoma	21	Recruiting
NCT06012734	Aug 2024	Ph.Ib	Atezolizumab	The Netherlands Cancer Institute	Colorectal cancer	37	Recruiting
NCT04560972	Oct 2024	Ph.I	Carboplatin, etoposide, atezolizumab	City of Hope Medical Center	Untreated extensive-stage small cell lung cancer	3	Active, not recruiting

improve checkpoint blockade in SCLC.[62] This work has paved the way for a partnership between Roche and academic researchers in which LB-100 is combined with either atezolizumab alone (NCT06012734, entry 6, Table 1), or with atezolizumab, carboplatin, and etoposide (NCT04560972, entry 7, Table 1) in Ph.I clinical studies.

Building on the foundational idea that LB-100 can reprogram the tumor immune microenvironment, Cui and coworkers (again with Zhuang and Lixte) investigated whether PP2A inhibition could also enhance the efficacy of chimeric antigen receptor (CAR) T cell therapy in solid tumors.[63] In murine models of glioblastoma and colon carcinoma, pre-treatment with LB-100 significantly boosted the antitumor activity of CAR-T cells targeting carbonic anhydrase IX (CAIX), a protein involved in HIF-1a hypoxic signaling.

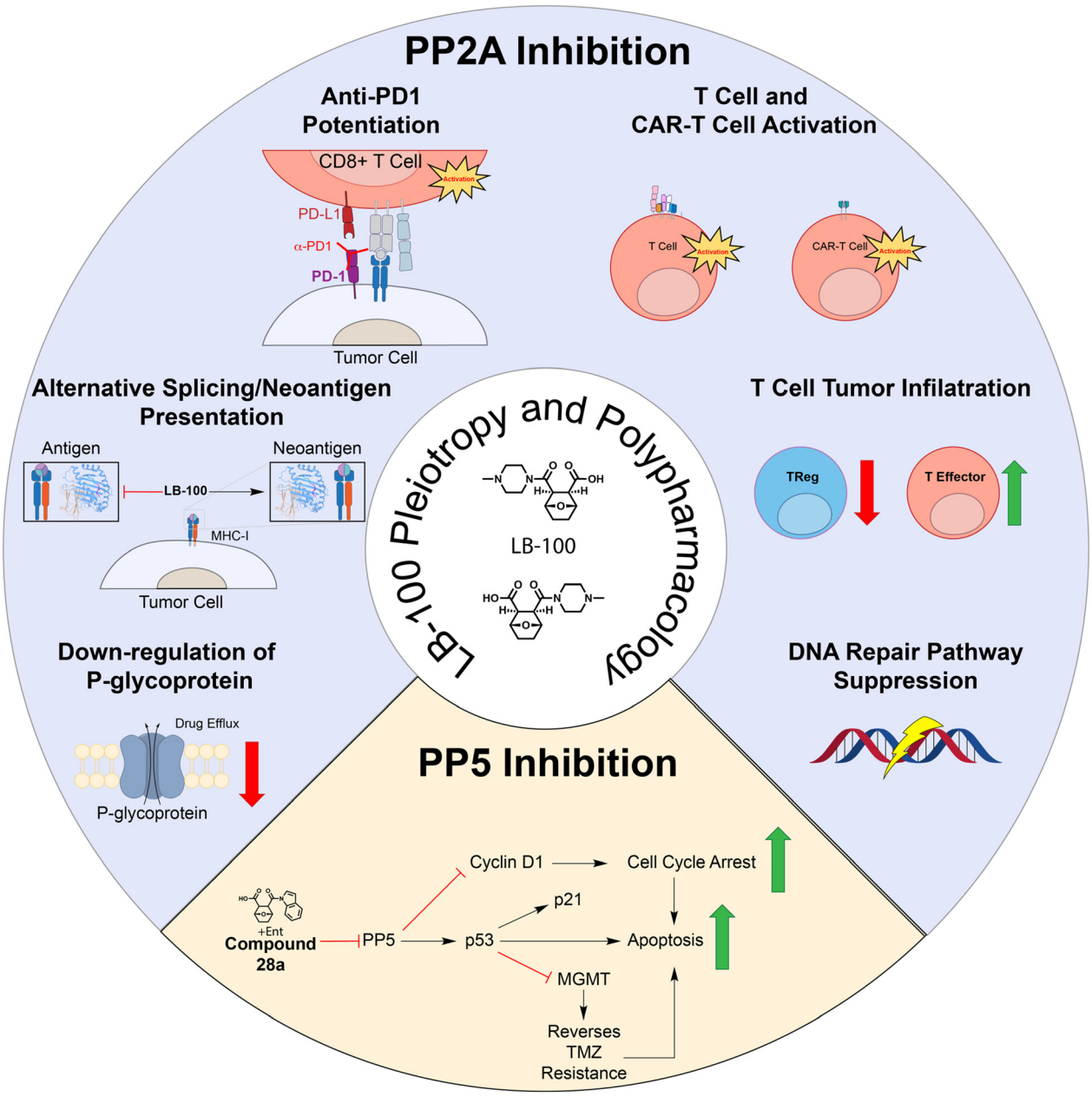


Fig. 9 LB-100 pleiotropy and Polypharmacology. Clockwise from top – top right: LB-100 has been shown to activate both native CD8 T cells and CAR-T cells; center right: LB-100 has been shown to decrease the number of Tregs in the tumor microenvironment and increase effector cell infiltration into the tumor microenvironment; bottom right: LB-100 inhibits PP2A leading to increased phospho-CHK1 and CHK2, suppressing DNA repair mechanisms; bottom center: an indole derivative of LB-100 (see Fig. 10) is selective for PP5, increasing cell cycle arrest and apoptosis, and reversing TMZ resistance; bottom left: LB-100 has been shown to decrease P-glycoprotein-mediated drug efflux; center left: by inhibiting PP2A in splicing mechanisms, LB-100 leads to alternative splicing of mRNAs, leading to presentation of neoantigen; top left: by inhibiting PP2A, LB-100 activates CD8+ T cells and potentiates both α-PD1 therapy in mice, and α-PD-L1 in both mice and humans, with the latter currently in clinical trials.

In light of the aforementioned studies, which show that LB-100 both upregulates PD-L1 expression and reprograms the tumor immune microenvironment by suppressing regulatory T cells and enhancing CD8+ cytotoxic T cell infiltration, emerging reports that PP2A inhibition with LB-100 also promotes neoantigen expression *via* alternative splicing are particularly compelling.[64,65] Taken together, these studies suggest that LB-100 may play a complicated role in immuno-oncology.

A recent study investigated the ability of LB-100 to help overcome multidrug resistance in aggressive cancers like glioblastoma and non-small cell lung carcinoma cells (NSCLCs).[66] In this study, LB-100 was tested in combination with adavosertib (a WEE1 kinase inhibitor) and doxorubicin in patient-derived cancer cells. LB-100 significantly enhanced the effectiveness of both drugs, not only after repeated treatments but also upon administration of a single dose. Importantly, LB-100 reduced the expression of P-glycoprotein (P-gp) – a key efflux pump which drives resistance. By lowering P-gp levels, LB-100 increased the accumulation and potency of doxorubicin, making previously resistant cancer cells more susceptible to treatment.[67] These findings suggest that LB-100 could be a valuable tool in re-sensitizing drug resistant tumors, particularly by targeting resistance mechanisms like drug efflux.

In 2019, D'Arcy and coworkers noted that, because LB-100 is likely to inhibit both PP2A and PP5, the effects of inhibiting each of these enzymes are different, LB-100's antitumor activity observed in clinical trials is likely due to additive effects.[39] Li an coworkers published a study showing that LB-100 readily hydrolyzes in water with only 14% remaining after 8 h and a half-life ($t_{1/2}$) of only 5 h in plasma (see Fig. 11 for details).[68] This raises the question of whether LB-100 is the active pharmacophore, or whether endothall/norcantharidin also has therapeutic effects in this arena, and the *N*-methylpiperazine of LB-100 is simply modifying physicochemical properties, making it a pro-drug.

LB-100 clinical trials

LB-100 is currently under investigation in clinical trials in combination with a wide range of cancer treatments, including docetaxel (trial completed), surgery (trial completed), immunotherapy, doxorubicin, etoposide, cisplatin, and radiation (Table 1). The mechanisms by which these treatments are purported to work in the clinic are somewhat ambiguous, and as we noted in the previous section. Given the pleiotropy and polypharmacology involved (Fig. 9), the true mechanisms may be quite complicated.

The therapeutic activity of LB-100 likely arises from its capacity to modulate multiple signaling pathways in concert, thereby broadening the therapeutic window. Among its key effects, PP2A inhibition prevents the deactivation of CHK1 and CHK2, kinases activated in response to DNA damage, as well as RAD51 and BRCA1, which are central to homologous recombination repair of double-stranded break. By suppressing these repair mechanisms, LB-100 enhances the cytotoxicity of agents such as topoisomerase inhibitors (etoposides), DNA crosslinkers (platinum compounds), DNA intercalators (doxorubicin), and reactive oxygen species-inducing agents. In this way, LB-100-mediated PP2A inhibition acts as a powerful potentiator of antitumor therapies.

Interestingly, LB-100 and related inhibitors exhibit a biphasic effect on T cells: low concentrations enhance T cell activation, whereas higher concentrations suppress it. This dual behavior aligns with PP2A's extensive role in dephosphorylating roughly half of the phosphoproteome, including critical nodes in T cell signaling pathways. It is also noteworthy that LB-100 is neither the most potent PP2A inhibitor within this chemotype family, nor the most selective. While this may seem counterintuitive in the broader context of drug discovery, the decision not to advance the most potent candidates suggests that, given PP2A's diverse and context-dependent functions, the "sweet spot" of partial inhibition was essential for achieving a balance between efficacy and tolerability.

Grade 3 adverse events associated with LB-100 administration include anemia, decreased creatinine clearance, dyspnea, hyponatremia, and lymphopenia.[69] As mentioned above, it is unclear whether LB-100 or norcantharidin/endothall is the pharmaceutically active component, and if it's LB-100, it is unknown whether one enantiomer is more active than the other.

PP2B (calcineurin) inhibitors

After PP2B (calcineurin) became known as a regulator of immune function, inhibitors of the protein were sought after as immune suppressants. The first observation that norcantharamides inhibit came from a 1997 report that compared norcantharidin and some of its analogs with cyclosporin A in assays measuring IL-2 secretion and β-galactosidase thymidine kinase RGA activity (Fig. 10a).[70] In this study, compound **42** (Fig. 10a) was shown to bind to PP2B with a K_i of 2.78 μM, which is a good starting point to develop a PP2B inhibitor. However, compound **42** was a more potent binder to PP2A, with a K_i of 0.18 μM, creating a selectivity problem. This result is somewhat surprising because these compounds have previously been known to inhibit PP2A, which is active in the Akt/mTOR pathways, and helps to keep T cells quiescent. Thus, one would expect PP2A inhibition to potentiate T cell activation (indeed, this was observed in the aforementioned studies). The group reported that dimethyl norcantharamide **42** inhibits PP2B-dependent dephosphorylation of a substrate peptide commonly used in PP2B assays. Furthermore, the same compound suppresses IL-2 secretion, a PP2B-dependent process, in Jurkat cells. These results demonstrate calcineurin inhibition by compound **42**.[70]

In the same year, Sodeoka and coworkers began exploring inhibition of PP2B using cantharidin analogs by using

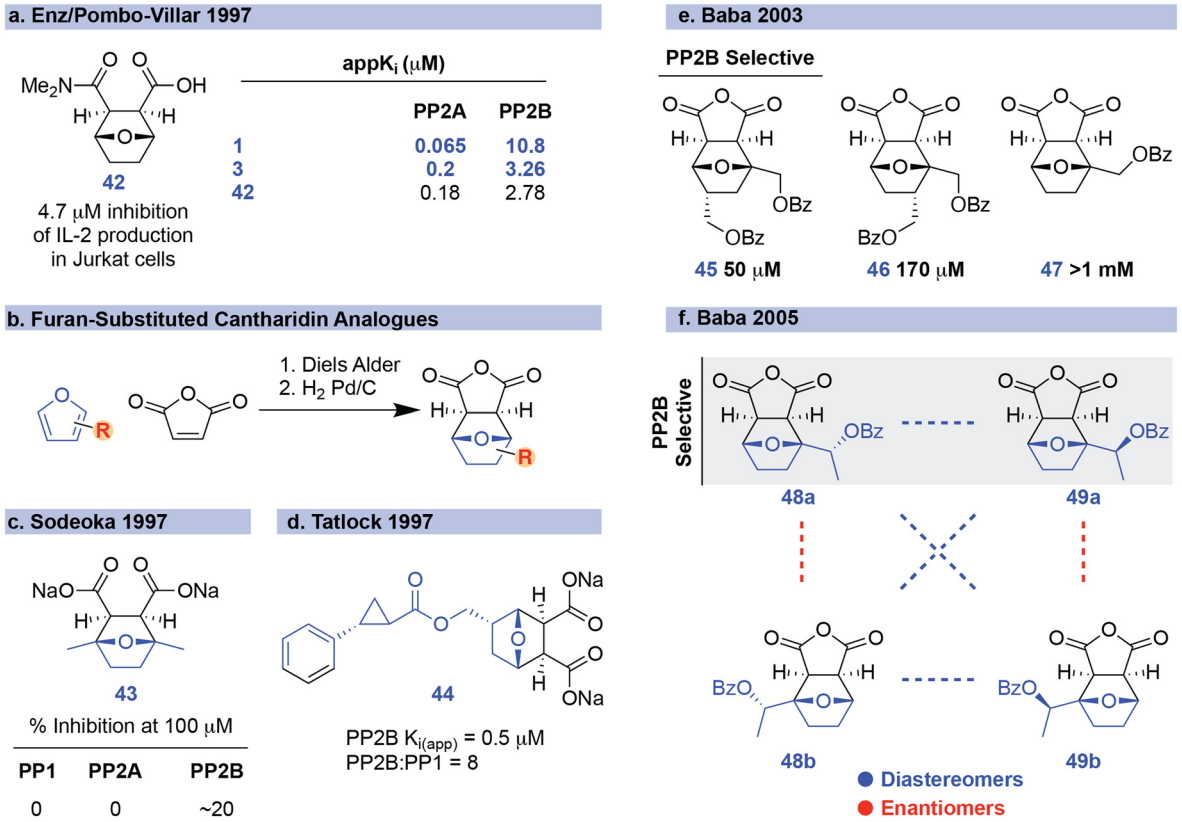


Fig. 10 PP2B-selective cantharidin-based scaffolds. a. The dimethylamide of norcantharidin was the first reported cantharidin derivative to exhibit relatively potent PP2B affinity, in the low micromolar range; b. substituted furans became the focus in developing selective cantharidin-derived PP2B inhibitors; c. the C1/C4 dimethyl substituted disodium salt of endothall (hydrolyzed norcantharidin) was one of the first cantharidin derivatives exhibiting PP2B selectivity, tough with low potency; d. a more elaborated C5 or C6 substituted analog was the first to show both potency (0.5 μM) and selectivity (16 fold for PP2B over PP1) or PP2B; e. further elaboration of the furan precursor yielded compounds with modest activity, though some had good selectivity, with compound 6 being nearly specific for PP2B; f. separation of enantiomers and diastereomers yielded insights into the shape of the PP2B binding pocket.

substituted furans in the Diels–Alder reaction with maleic anhydride (Fig. 10c).[40] In addition to cantharidin, the team found 12 other compounds that inhibited PP1 and PP2A at concentrations of 1 mM or 100 μM, and PP2B to varying extents at the same concentrations. However, the team found that the disodium salt of 1,4-dimethylnorcantharidin diacid (1,4-dimethylendothal disodium, compound **43**) inhibited PP2B by around 20% at or above concentrations of 100 μM, but did not inhibit PP1 or PP2A at all at those concentrations. While these are poor binding affinities for a lead compound, a mechanism for selective binding to PP2B over PP1 or PP2A by substitution at C1 and C4 (as well as C5, the authors note) represented progress toward the goal of generating potent and selective phosphatase inhibitors based on the (nor)cantharidin scaffold.

Tatlock and coworkers followed this work with a structure-based approach to develop a more potent cantharidin analog targeting PP2B. The group using furans modified in multiple positions to generate C1, C4, C5 and/or C6-substituted analogs. The greatest potency coming from an acetoyl group in position C5 (compound **44**, Fig. 10d).[38]

In 2003, Mikiko Sodeoka and Yoshiyasu Baba continued the work on substituted furan Diels–Alder reactions for making modified cantharidin analogs.[71] This work further advanced insights into SAR of PP2B binding, with a selective 50 mM inhibitor (compound **45**) and two less potent and unselective compounds (**46** and **47**), setting the rules for C1/C4 and C5/C6 substitution for PP2B engagement (Fig. 10e). Two years later, the team went on to introduce a stereocenter on the C1/C4 sidechain, creating a set of enantiomers and diastereomers, which they separated and tested (compounds **48a**/**b** and **49a**/**b**, Fig. 10f).[72] These data give insight into the shape of the PP2A and PP2B active sites.

PP5 inhibitors and the elimination problem

Studies have shown that while LB-100 is somewhat selective for PP2A, the compound also inhibits PP1 and PP5 (Fig. 11a).[39] The phosphatase PP5 happens to be an attractive target for treating certain cancers due to its role in negative regulation of the tumor suppressor p53. Inhibiting PP5 can increase levels of active p53 which can lead to a reduction in tumor size. Furthermore, PP5 has

relatively few substrates (21 *vs.* PP2A's 86) so therapeutic targeting of PP5 may have fewer unwanted downstream.[73,74] To this end, Li and coworkers set out to develop a compound more stable in aqueous solution than LB-100 and targeting PP5.

The team did a thorough investigation of LB-100 in aqueous solution and plasma and found that the compound rapidly degrades *via* an anhydride ring-closing mechanism, eliminating the *N*-methylpiperazine. One of us (KAS) observed similar elimination mechanisms which precluded us from using a piperazine as an attachment point for functional handles (unpublished work). Li and coworkers designed and tested a panoply of nitrogen heterocycle derivatives to overcome the stability problem[68] (Fig. 11b).

The resulting compound **50** was >20 fold selective for PP5 over PP1, and >30 fold selective for PP5 over PP2A. Furthermore, compound **50** increased p53 and p21 levels, decreased cyclin D1 levels, and reversed temozolomide resistance in U87 MG cells. The compound also has better physicochemical properties than LB-100 (notably a clog*P* of 2.4 *vs.* LB-100's −1.4). Finally, stability was much improved over LB-100. In head to head assays, LB-100 was either completely or almost completely hydrolyzed after 24 hours while 98.3% of compound **50** remained in water, 92.0% in simulated gastric fluid, and 96.0% in simulated intestinal fluid. Overall, this study demonstrated that PP5 selectivity, physicochemical properties, and stability can be improved by modifying the amide heterocycle on such analogues.

Anhydride ring opening and PPP-binding mechanism

In 1997, Sodeoka observed that the X-ray crystal structure (PDB: 1fjm)[75] of microcystin bound to PP1 revealed that "an interaction between the two carboxylic acid groups of microcystin" with two metal atoms at the active site of PP1 suggest that "the two carboxylic acid substituents in cantharidin also interact with the active site" of phosphatases to which it binds.[40] In 2000, McCluskey noted that "only analogues capable of undergoing facile ring-opening of the anhydride moiety displayed any significant inhibition".[37] These observations lead us to speculate that the dicarboxylate binding mechanism extends to all cantharidin-based structures that bind selectively or non-selectively to various PPP members. This interaction is shown using a computational model of cantharidin bound to PP2A in Fig. 12a.

In 2019, D'Arcy and coworkers published a crystal structure of LB-100 bound to PP5C.[39] Although this structure (PDB: 5wg8) shows the LB-100 hydrolysis product, (endothall, the diacid form of norcantharidin), the crystal structure demonstrates that the bridgehead oxygen cooperates with the two carboxylic acids to bind to the manganese atoms at the active site of the protein (Fig. 12b). This structure confirms the earliest SAR observations by McCluskey that the 7-O bridgehead is important for binding. To date, this is the most robust evidence that the cantharidin diacids coordinate with the active site metals of PPP phosphatases.

A 2019 study by Meng Choy with Rebecca Page and Wolfgang Peti corroborated the microcystin-LR binding mechanism using PP1 H66K but with a greater resolution of the carboxylic acid of microcystin LR which is engaging with a single metal atom, PDB: 6obq.[76] This structure is compared with the earlier Sodeoka structure in Fig. 12c.

Sodeoka's conjecture that cantharidin and microcystin LR may share qualities of binding mechanism brings to mind other natural product phosphatase inhibitors that have a similar diacid moiety, particularly ones which have the diacid

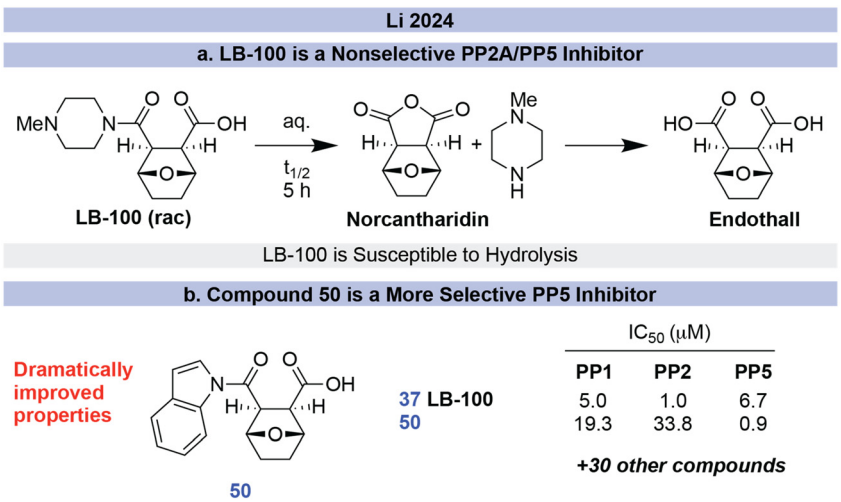


Fig. 11 Indoleamine variation on LB-100 for PP5 selectivity. a. Li and coworkers showed that LB-100 readily hydrolized in aqueous media, forming endothall and methylpiperazine, raising questions about the pharmaceutically active component; b. the same group showed the LB-100 is not selective for PP1 or PP2A, but also inhibits PP5. Using this information, the group made dozens of analogs to make the scaffold more than 20-fold selective for PP5 over PP1 and PP2A.

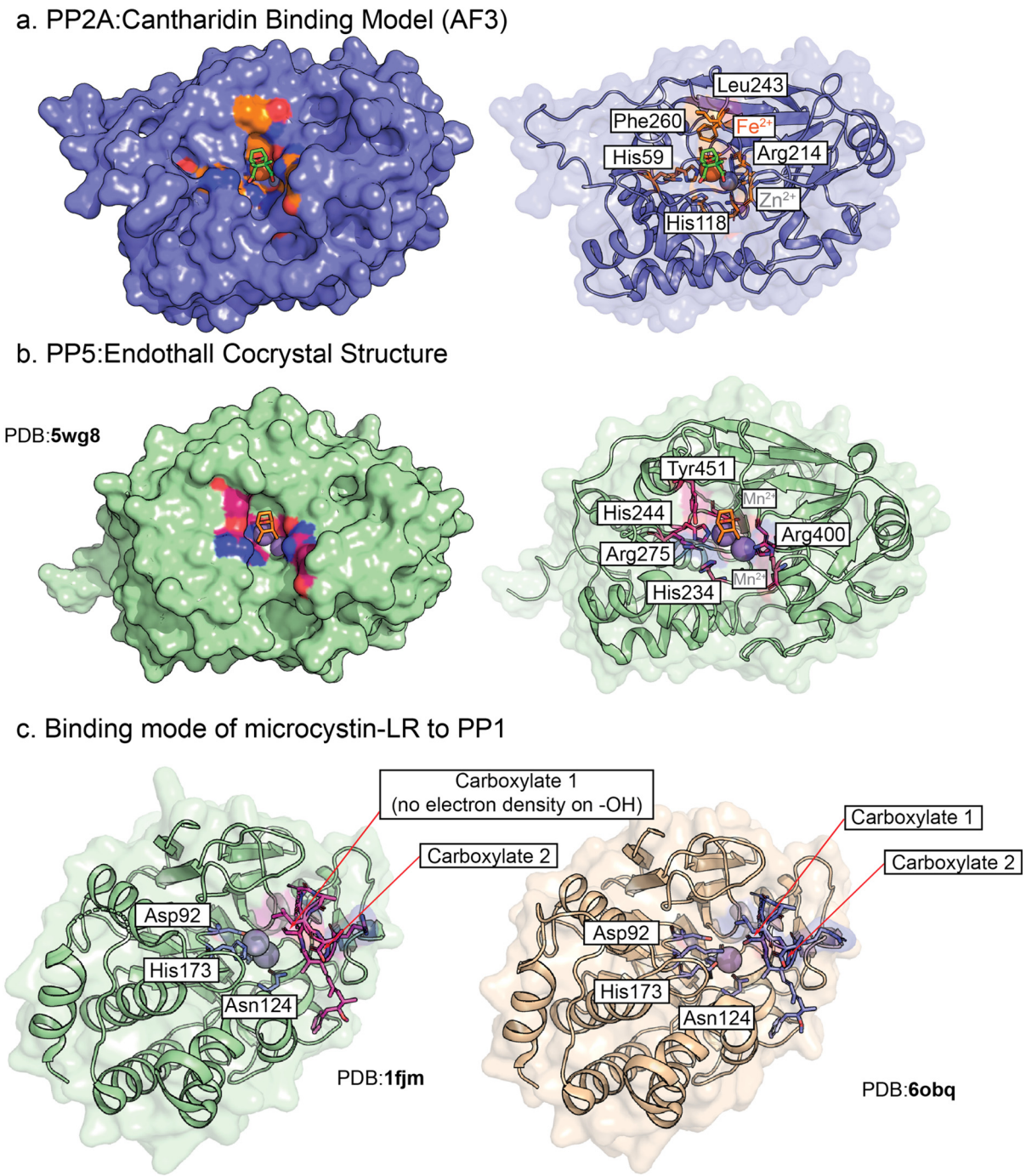


Fig. 12 Computationally-derived and experimental models of cantharidin-based structures indicate that binding to the active site of PPP phosphatases is mediated by carboxylate coordination with active-site metals. With several published phosphatase structures, a crystal structure of the LB-100 hydrolysis product endothall, and computational methods such as AlphaFold3 (AF3),[77] modeling of active site ligands with Mn/Mn or Fe/Zn active site metals can be useful for designing new analogs. Structures were prepared using AlphaFold3 webserver, PyMol3, and Schrödinger Maestro (see Fig. 13 for clear microcystin-LR structures). a. Binding of cantharidin to PP2A is modeled using an AF3 model of PP2A with manganese atom and one iron atom at the active site, then overlayed with the crystal structure of endothall bound to PP5 (PDB:5wg8), and subsequent alignment of cantharidin with the endothall partial structure, before minimizing structure energy with Schrödinger Maestro; b. crystal structure of PP5 bound to endothall with two manganese atoms at the active site (PDB: 5wg8); c. crystal structures of PP2A bound to native PP2A (left) and PP2AH66K (right), which can only bind one metal atom, showing that like cantharidin, mycrocystin-LR uses coordination of two carboxylates with the active site metals (in this case two or one manganese atoms, respectively), suggesting similarities in binding mechanism across diverse structures.

in a conformation suggestive of possible anhydride formation like cantharidin, or a phosphate group (Fig. 13). The negative charge density of the diacid is reminiscent of the phosphate groups that typically bind to the active site.

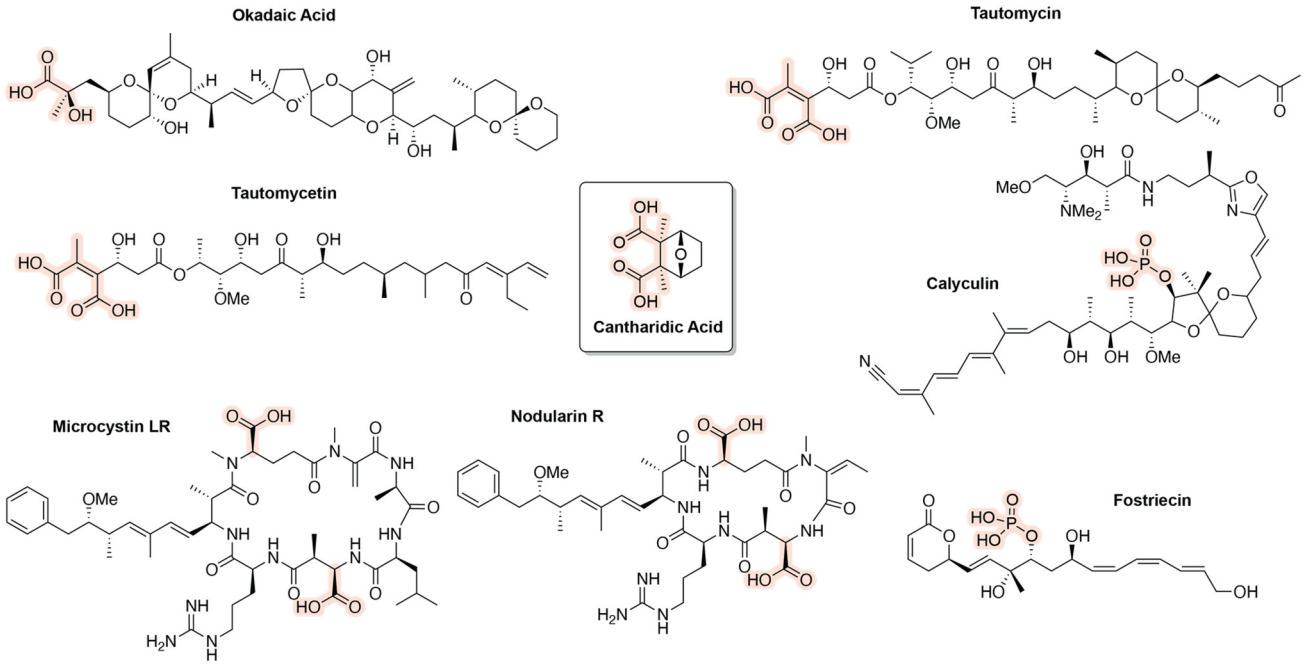


Fig. 13 Cantharidin in its active binding form (cantharidic acid) has similar dicarboxylate binding elements to other well-studied natural product phosphatase inhibitors.

As a final note on binding mechanism, it is known that at least in some cases, the active site metals of PP2A are iron and zinc, and that these metals are often displaced by metals of a similar valency during purification for *in vitro* assays, often manganese. It may be worth taking into consideration the difference in Lewis acidity between these metals in *in vitro* screening campaigns. In this case, manganese is the strongest Lewis acid, followed by zinc and then iron. Using hard/soft acid base theory, one would expect that the strongest interactions would be between cantharidin-based diacids and manganese, with weaker interactions between the diacids and the iron/zinc pair. These differences may lead to discrepancies between *in vitro* results and cellular or organismal observations, although more work is required to establish these claims.

YCANTH and cantharidin as a topical

Cantharidin has a long history of dermatological use, particularly for the removal of warts and other benign skin lesions. Although it was widespread in clinical use by dermatologists prior to the formation of the FDA, it had never undergone the formal approval process that had later become standard. When the FDA was established, cantharidin was effectively "grandfathered" in as an available treatment based on its pre-existing use. However, in 1963, the FDA banned its dermatological use after manufacturers failed to provide sufficient evidence of efficacy in well controlled clinical trials, as required under the 1962 Kefauver–Harris Drug Amendments.[1] This prohibition lasted for decades, despite continued off-label or compounded use by some dermatologists.

In a notable regulatory reversal, cantharidin gained its first official approval in 2023 under the brand name YCANTH, marketed by Verrica Pharmaceuticals, Inc,[78] indicated for the topical treatment of molluscum contagiosum in patients aged 2 years and older. This approval marked a significant milestone, not only validating decades of anecdotal clinical experience, but also demonstrating that modern, rigorously conducted clinical trials could establish both the efficacy and safety of cantharidin for a clearly defined dermatological condition.

Select patent space

The patent literature on cantharidin derivatives reflects ongoing efforts to refine PP2A (and to a lesser extent, PP5 and PP2B) inhibition for therapeutic use. Strategies include modifying the chemical scaffold to maintain high affinity for PP2A catalytic subunits while reducing off-target phosphatase inhibition, improving pharmacokinetics, and enabling target delivery. A major gap in the patent space involving the therapeutic use of cantharidin derivatives resides in C2/C3 substituted analogs with unique carbon and heteroatom sidechains. We expect that an expansion of available chemistries will not only open the door to improved potency and target selectivity, but also to uncharted patent space.

In 2005, Salvati and coworkers at Bristol Myers Squibb screened a modest library of 350 compounds in search of androgen receptor (AR) agonists and discovered a family of compounds including isoindoledione **51** (Fig. 14a).[79] Modeling and iterative drug design led to the [2.2.1]-oxabicyclo imide-derivative **52**, in which the methylene bridgehead was modified to an oxygen bridgehead, resembling the (nor)cantharidin core structure.[80] The potent and selective AR agonist **53** (BMS-641988) was developed through optimization of 52 and investigated for the treatment of prostate cancer.[81]

Scores, if not hundreds of molecules have been disclosed. In the broader context of these cantharimide structures in search of phosphatase inhibitors, especially prior to the understanding that one or two free carboxylates are required for PPP active site binding. Most of them are not covered in this review because they are not active against PPPs. We chose to highlight the BMS compounds for two reasons: 1. using (nor)cantharidin to target nuclear hormone receptors represents a departure from the PPP field of study, and 2. reactions disclosed in a patent covering these structures may offer a new inroad to C2/C3 substituted cantharidin analogs, which may be useful for generating novel PPP inhibitors.[82]

The first of these reactions introduces a novel fused cyclic ether by reduction of one of the imide carbonyls of **54** before acid catalyzed cyclization to give **55** (Fig. 14b). The alpha acylation to give **56** (Fig. 14c) is a particularly interesting transformation which, after hydrolysis of the aryl imide and subsequent anhydride formation could, in theory, give rise to a whole new structural class of PPP inhibitors, though this investigation has yet to be realized.

Other cantharidin-based scaffolds

A few other structures are worth mentioning here, despite lacking phosphatase activity, and are not discussed in detail or given unique numbers. These compounds are listed here to highlight the (nor)cantharidin chemical space that has been explored.

In addition to laying much of the foundational work on norcantharidin analogs in the early 2000s, McCluskey and coworkers were quite prolific in generating new interesting structures that are based on the cantharidin core structure (Fig. 15a–c).[83–86] Fused lactone and lactam derivatives from McCluskey's Newcastle group also represent interesting constrained scaffolds, introduced in 2003 (ref. 48) and 2019,[50] respectively, though they generally lack PP2A activity (Fig. 15d). Other interesting cantharidin-like compounds have been synthesized based on scaffolds isolated from biomasses, and contain interesting fused lactone and spiromide moieties (Fig. 15e).[83]

Other commendable efforts toward cantharidin analogues have resulted in advanced delivery systems and specialized formulations,[87–89] as well as cantharidin–drug conjugates.[88–90] The latter involve norcantharidin anhydride ring-opening with nucleophilic amines of existing approved

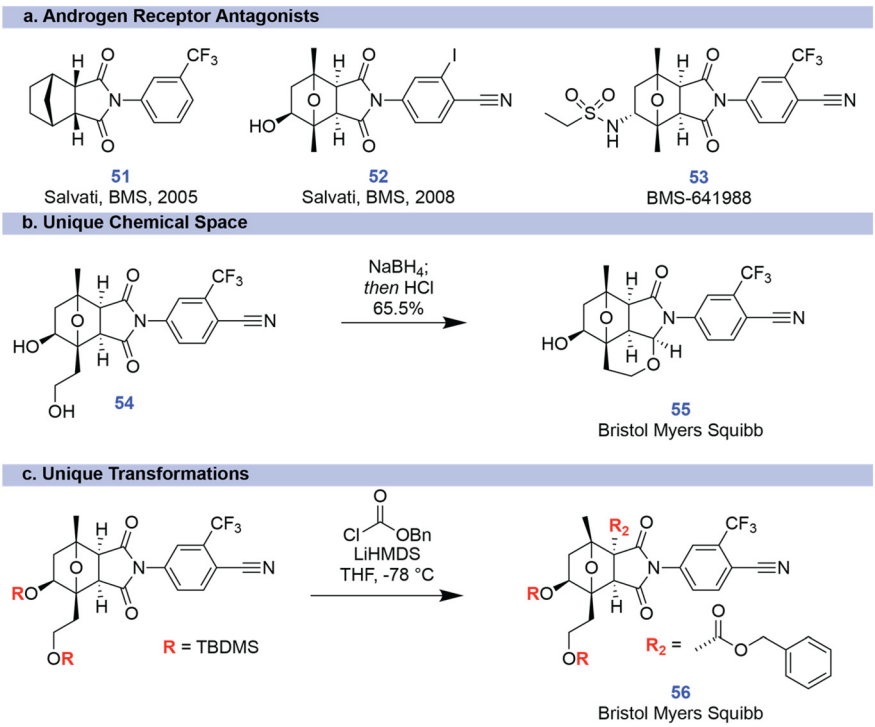


Fig. 14 Other cantharidin-like scaffolds. a. In the early 2000s Bristol–Myers Squibb discovered a cantharidin-like scaffold in search for AR Antagonists, which they subsequently optimized; b. novel chemical space that came out of these studies include an interesting pyranoisoindole; c. formation of the enolate of norcantharimide scaffolds can be acylated with benzylchloroformate.

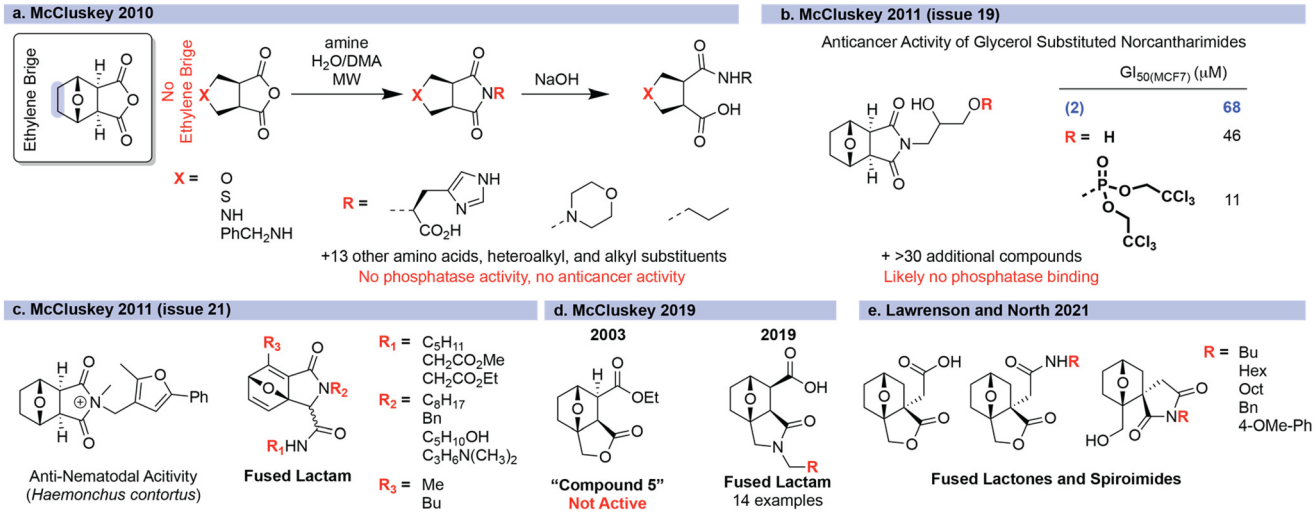


Fig. 15 Miscellaneous cantharidin-like structures. a. Interesting scaffolds lacking the ethylene bridge of the norcantharidin core did not exhibit phosphatase activity, likely due to lack of coordination with the active site metals; b. glycol derivatives including phosphate esters showed good antiproliferative activity but are likely not active toward phosphatases; c. quaternary imides with nematodal activity, as well as substituted fused lactams, are interesting norcantharidin derivatives but lack phosphatase activity; d. some interesting fused lactone and lactam had no phosphatase activity (from studies first described in Fig. 7d and 8d); e. some fused lactones and spiromides fit into this category, though these were never investigated for biological activity.

drugs, and may prove to be a clever approach, especially if the resulting amide is hydrolized the way LB-100 is hydrolized (revisit Fig. 11a), resulting in norcantharidin and the drug in question being released upon entering the cell. However, while clearly warranting attention, extensive review of these technologies is beyond the limited scope of this review.

Targets beyond PP2A

While protein phosphatases PP1 and PP2A remain the most well-established intracellular targets of cantharidin and its derivatives, PP5 is also an established target. There is growing evidence that these molecules modulate additional pathways and molecular targets that may be therapeutically relevant. The downstream consequences of PP2A inhibition are far-reaching because PP2A regulates the phosphorylation status of numerous signaling proteins. For example, alterations in MAPK signaling and DNA damage response pathways (including Chk1/Chk2) observed in cantharidin-treated cells are not the result of direct binding to these kinases, but rather emerge from the sustained phosphorylation of upstream or regulatory components normally dephosphorylated by PP2A. Similarly, PP2A's influence on NF-kB pathway components and other transcriptional regulators means that cantharidin-induced changes in gene expression, including cytokine profiles that can modulate immune checkpoint expression, are also indirect. This relatively narrow phosphatase profile, coupled with wide-ranging downstream effects, offers both an opportunity for therapeutic specificity and a challenge in predicting the full biological impact.

PP2B (calcineurin) has long been a target of interest for suppressing the immune system to treat autoimmune disorders and organ transplant rejection and many other inflammatory disorders. Cantharidin analogs could expand the toolkit of treatments currently represented by best-in-class drugs like tacrolimus and cyclosporin A, potentially offering more direct targeting of PP2B (tacrolimus binds to FK506 and inhibits PP2B indirectly) with a dramatically simplified structure.

Historically, a major hurdle preventing selective targeting of PPPs and other relevant proteins has been the lack of structural data required for structure guided design. The recent publication of crystal structures together with major advances in computational methods like AlphaFold3 and ultra-high throughput screening methods may accelerate the discovery of novel cantharidin analogs for targeting PPPs, particularly with the consolidation of SAR understanding and new methods for C2/C3 substitution. After reviewing the entirety of the cantharidin literature suggests that diversifiable C2/C3-substituted may be not only more potent than current clinical candidates but can also reach outside of the highly conserved PPP active sites to coordinate with residues that are more unique to individual PPP family members.

Conclusion

Cantharidin and its derivatives continue to attract attention as reasonably potent inhibitors of serine/threonine protein phosphatases with excellent opportunity for diversification. PP2A has been a major focus of the efforts in this arena, though structural manipulations can confer selectivity for PP2B and PP5 as well (PP2A-targeting analogs probably also

inhibit PP1). Future structural studies may enable derivatives selective for the remaining members of the family.

PP2A-targeting derivatives like LB-100 have demonstrated notable potential as anticancer agents. The mechanisms of action are complicated, though at least part of their activity disrupts key phosphatase-regulated signaling pathways, leading to effects on cell cycle progression, apoptosis, and tumor growth. Furthermore, it seems that LB-100-mediated PP2A inhibition has an unforeseen benefit in that it stimulates tumor infiltration of cytotoxic T lymphocytes and suppression of regulatory T cells. This has spurred two recent clinical trials in which LB-100 is co-administered with PD-1-targeting antibodies.

Targets beyond PP2A have had comparatively less success – yet. Members of the PPP family of phosphatases to which PP2A belongs are highly conserved and play a role in practically every biochemical pathway. As such, there may be significant potential for using cantharidin derivatives to target these enzymes in much the same way that LB-100 has for PP2A, though this will require new chemistries to access C1/C2 substituted analogs.

The unique, information-dense core structure of the cantharidin scaffold has overcome significant toxicity issues and proven its potential to be used as a systemic therapeutic. Further development of innovative chemistries may broaden the therapeutic landscape that can be impacted by scaffold derivatives. Given the ubiquity of the PPP family of phosphatases in diverse pathologies, and cantharidin's unique potential to inhibit members of this family, we anticipate that many new therapeutic opportunities can be uncovered by exploring this space.

Author contributions

KAS made all of the figures. KAS and AM wrote the manuscript. JTN guided the synthetic analysis of total syntheses. WW provided synthetic, medicinal chemistry, and oncology expertise.

Conflicts of interest

There is no conflict of interest to declare.

Data availability

This article is a review and does not include any new experimental data. No original datasets were generated or analyzed for this work. All models discussed within the article are conceptual or structural representations derived from previously published protein structures available in the Protein Data Bank (PDB). These models were constructed solely for illustrative and comparative purposes, based on publicly accessible structural data that adhere to FAIR (Findable, Accessible, Interoperable, and Reusable) principles.

Readers interested in reproducing or further exploring the models presented in this review may obtain the underlying PDB entries directly from the Protein Data Bank (https://www.rcsb.org). Annotated model files and related visualizations used in the preparation of figures are available from the corresponding author upon reasonable request.

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