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Following the Lead from Nature with Covalent Inhibitors

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Abstract: Covalent inhibitors are re-emerging as pharmacologically interesting entities with several candidates having received recent approval for therapeutic intervention. Nature has embraced this strategy and many natural products possess mildly electrophilic moieties able to covalently engage a target protein. This review surveys recent case studies for the identification of the target proteins of natural products. While sesquiterpene lactones represent a vast repertoire of covalent inhibitors, they can also be found in other classes of natural products, with sometimes unusual mechanisms to unmask the electrophilic moieties. These examples ought to be inspiring for the development of new biochemical probes and tomorrow's first-in-class drugs.

Keywords: Covalent inhibitors · Mode of action · Natural products · Protein modification · Target identification

1. Introduction

Covalent inhibitors have long been met with scepticism from the medicinal chemistry community, owing to the risk of idiosyncratic immune responses and liver damage due to promiscuous and unselective reaction with host proteins.[1] Allergies to beta-lactams and the hepatotoxicity of the benzoquinimine metabolite of paracetamol have supported these concerns, and past drug discovery efforts have most often steered away from candidates bearing electrophilic functional groups. A number of success stories however served as a reminder that safe covalent inhibition is possible. To only name a few, aspirin, used for the treatment of inflammations, pain and fever, or omeprazole, for the treatment of gastrointestinal reflux and ulcer, are both on the World Health Organization's List of Essential Medicines, and to date, several dozens of covalent inhibitors are on the market for various indications, including infections, cancer, gastrointestinal and CNS disorders.[2,3]

The ongoing paradigm shift with respect to covalent inhibitors has been facilitated by a better understanding of target selectivity and engagement. Briefly, this selectivity is due to the pre-organization of the electrophilic trap in the inhibitor and a nucleophilic residue in the target following a non-covalent binary complex between drug and target. In a selective covalent inhibitor, this pre-organization leads to significantly faster covalent engagement than for a random, non-specific interaction. This covalent bonding essentially leads to a long if not infinite residence time which correlates well with drug efficacy, particularly in cases where the drug competes with an abundant or high affinity endogenous ligand. In addition, covalent targeting can allow discrimination of closely related targets if one bears an unconserved cysteine. Inhibition is resolved only upon target turn-over.

This is a highly appealing strategy and it prompted efforts for the discovery of novel covalent modifiers, which currently include three main approaches:[3–6]

The structural bioinformatics-based design of selective covalent inhibitors is one of the major approaches and largely benefits from a wealth of crystallographic data on X-ray crystal structures of protein–inhibitor complexes.[7] Additionally, sequence alignment of members of a protein family such as kinases and cysteine mapping allow the determination of unconserved cysteine residues in the binding site, which can in turn be covalently targeted by a suitably positioned electrophilic trap on the main pharmacophore.[8] Indeed, while the pharmacophore may bind reversibly to various members, covalent interactions will only take place in a smaller subset, thereby providing an additional selectivity filter.

The virtual screening of covalent modifiers has recently benefited from advances in the development of docking softwares allowing for covalent docking.[9] Thus, libraries of potential covalent inhibitors can be screened *in silico*. This approach has been elegantly exemplified with the identification of a number of novel covalent inhibitors for kinases such as JAK3, MSK1 and RSK2 with high potencies ranging from submicromolar to low-nanomolar *in cellulo*.

Large libraries of compounds can also be physically screened for the discovery of novel covalent modifiers. To this end, the screening of DNA-encoded libraries[10] of potential covalent modifiers can provide a rapid entry to hits by co-incubation with a target of interest. An alternative to DNA-encoding is the use of PNA-encoded libraries for display on DNA microarray,[11] which has allowed the discovery of covalent inhibitors for bromodomains such as PCAF[12] as well as kinases MEK2 and ErbB2.[13] This approach was shown to discriminate between high affinity non-covalent ligand and covalent ligand.

A very powerful approach developed by the Cravatt lab uses a library of fragments that is screened against the proteome of live cells. Specific ligand–protein interactions are identified by mass spectrometry. While this is possible for only a small set of fragments, live cells are the most relevant 'library' of potential targets. Fragments showing a selective target engagement can be refined by further functionalization.[14,15]

Before the advent of these modern discovery methods for covalent inhibitors, Nature had widely embraced this strategy, and many natural products contain mildly electrophilic moieties, such as beta-lactones and beta-lactams, epoxides or

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Michael acceptors, and they have provided a formidable source of inspiration for the development of covalent drugs by structural optimisation or adaptation of some unusual warhead mechanisms,[16,17] which is facilitated by advances in chemical proteomics for identification of their cellular targets.[18] We review some of the latest developments in the characterization of covalent natural product inhibitors, as well as unusual reaction mechanisms recently uncovered in natural products. This review focuses on covalent trapping of non-catalytic residues, and thus mechanism-based inhibitors or suicide-inhibitors are not covered.

2. Sesquiterpene Lactones

The sesquiterpene lactones are a large family of natural products with a high propensity of electrophilic traps capable of covalently engaging cysteine residues in target proteins (Fig. 1). They frequently bear an electrophilic α-*exo*-methylene moiety, with additional Michael acceptors or epoxides within their carbon skeleton or in their side chains. The past decade has brought sesquiterpene lactones under the spotlight for their engagement of targets relevant to the treatment of cancer and inflammation as well as infectious diseases.[19–21]

As the main active ingredient of herbal extracts of the medicinal plant Feverfew (*Tanacetum parthenum*), parthenolide (Fig. 1A) is one of the sesquiterpene lactones that has received the most attention. Investigations into its pharmacological profile revealed that parthenolide is an inhibitor of the NF-κB pathway, key regulator of the expression of genes notably involved in inflammatory processes and cancer.[22] While the modulation of the NF-κB pathway by parthenolide seemed to occur upstream from NF-κB inhibitor protein IκBα in the signalling pathway, the exact target and mode of action were unknown. Using parthenolide and a parthenolide-biotin conjugate, Crews and coworkers demonstrated that parthenolide inhibits the TNFα-stimulated NF-κB pathway. Using electrophoretic mobility shift assays, parthenolide was found to covalently engage IKKβ, responsible for the phosphorylation of IκBα and concomitant NF-κB activation. Parthenolide possesses two electrophilic functional groups, *i.e.* an α-*exo*-methylene-γ-butyrolactone and an epoxide. Reduction of the α-*exo*-methylene completely abolished parthenolide's anti-inflammatory activity *in vitro* and *in vivo* and did not prevent binding of the biotinylated probe to IKKβ in competition assays, thus establishing the mode of covalent engagement. Finally, a complete loss of biological activity was observed with the C179A IKKβ mutant, which allowed the authors to conclude that parthenolide binds to IKKβ at cysteine 179.[23] The poor pharmacokinetic profile of parthenolide for potential clinical applications, including low water solubility and bioavailability, led the Crooks lab to develop the LC-1 prodrug containing an *N*,*N*-dimethylamino moiety, which resulted in 1000-fold increase in water solubility, and it is currently being investigated notably for the treatment of acute myelogenous leukaemia[24] and drug-resistant glioblastoma.[25]

Deoxyelephantopin is a sesquiterpene lactone with a heavily functionalized germacranolide skeleton (Fig. 1B). It is the main herbal ingredient of herbal extracts from *Elephantopus scaber* used in traditional medicine.[26] It is relatively abundant and was found to be superior to taxol in suppressing orthotopic murine breast cancer, raising questions about its mode of action.[27] It was also found to suppress proteasome activity[28] and to inhibit the NF-κB pathway.[29] Furthermore, SPR experiments suggested that deoxyelephantopin reversibly binds to the nuclear receptor PPARγ in its ligand-binding pocket. This however contains a cysteine residue known to covalently engage oxidized fatty acids;[30] furthermore, the timeframe of SPR experiments may be too short to detect the formation of covalent bonds. In order to address these questions, our group engaged in a synthetic campaign, which provided a library of structural and alkyne-tagged analogues.[31] The cytotoxicity of deoxyelephantopin was found to be due to caspase-mediated apoptosis, and proteomic studies allowed us to identify 11 new covalent cellular targets, three of which, *i.e.* CBS, CTTN and CSTB, are known to cause cell death upon depletion. Furthermore, we demonstrated that deoxyelephantopin is a covalent PPARγ antagonist, with its α-*exo*-methylene engaging cysteine 176 in the zinc-finger domain and not in the ligand-binding pocket as anticipated. We were also able to identify a more potent PPARγ binder from our library. These results stand out as it is the first covalent inhibitor shown to engage cysteine

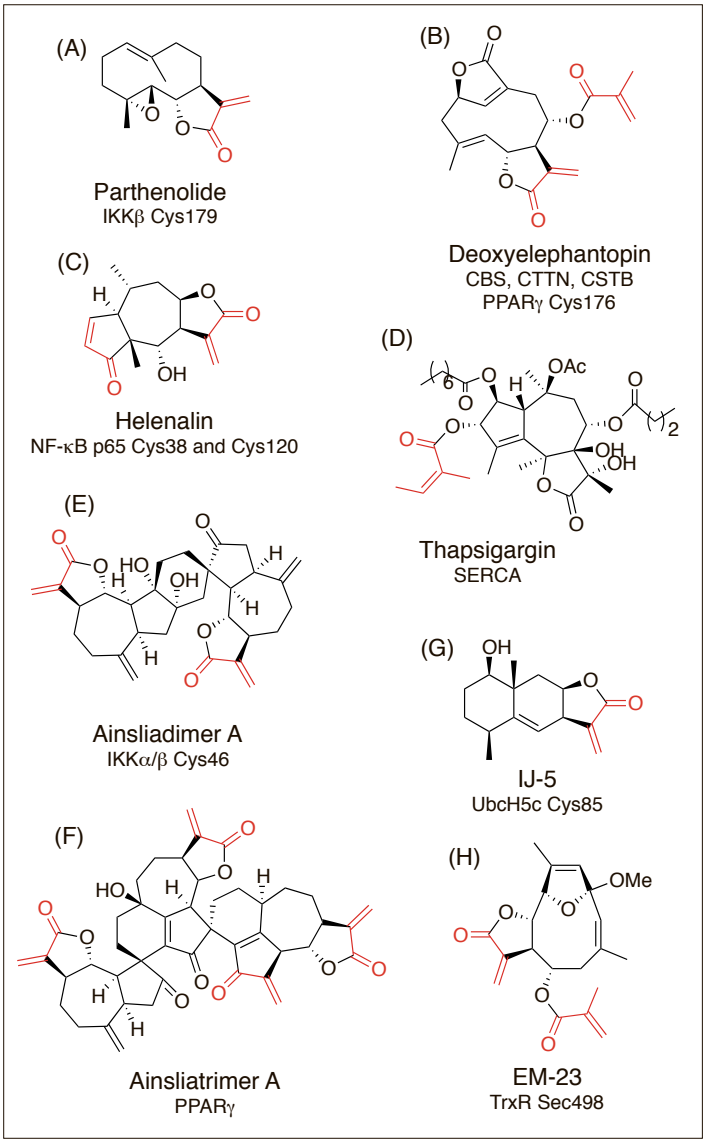


Fig. 1. Selected sesquiterpene lactones with their target protein and the modified residue.

residues in a zinc-finger domain, which in turn directly interferes with the high affinity PPARγ-DNA interaction. Furthermore, PPARγ being one of the most downstream enzymes in this signalling pathway, directly targeting it would prevent off-target effects.

The guaianolide and pseudo-guaianolides are another important subset of biologically active sesquiterpene lactones. Helenalin is a pseudo-guaianolide and the main active ingredient of tinctures of *Arnica* and *Helenium* species used in traditional European medicine as an anti-inflammatory (Fig. 1C).[32] Helenalin possesses an α-*exo*-methylene-γ-butyrolactone and a cyclopentenone, which are both electrophilic, and medicinal chemistry efforts have demonstrated that helenalin covalently reacts with cysteine 38 and potentially with cysteine 120 in p65, which forms the NF-κB heterodimer together with p50. This alkylation, and potentially cross-linking, at the DNA-binding interface of p65 sterically hinders DNA binding, thereby preventing the transcription of NF-κB-dependent genes.[33,34] Being able to directly inhibit NF-κB is very appealing as many of the upstream enzymes in the canonical NF-κB signalling pathway are involved in other pathways, and targeting them would lead to higher specificity and avoid off-target effects. The availability of helenalin from natural sources is limited. Harki and coworkers designed simplified helenalin analogues, whereby the seven-membered ring is removed.[35] These analogues are essentially obtained in three steps from readily available starting materials and recapitulated the biological activity of the natural product, as demonstrated using cellular luciferase reporter assays as well as labelling studies and mass spectrometry-based proteomic assays with alkyne-tagged probes. While this opens therapeutic opportunities in targeting NF-κB, it also shows that close analogues of natural products bearing essential pharmacophores for covalent engagement can be as potent and specific as the parent natural products thanks to the added selectivity offered by covalent interactions. A library of sesquiterpene-inspired analogues also provided alternative pharmacophores for NF-κB inhibition.[36]

Thapsigargin is the main active ingredient of *Thapsia garganica*, a medicinal plant endemic to Europe (Fig. 1D). With its heavily oxidised and functionalised carbon skeleton, this guaianolide doesn't include the reactive α-*exo*-methylene moiety typically involved in covalent interaction with cysteines, as opposed to the other sesquiterpene lactones discussed herein, but it possesses an angelate side-chain, which is electrophilic. Thapsigargin was found to stoichiometrically and irreversibly inhibit the sarco/endoplasmic reticulum Ca^{2+} (SERCA) family of ATPases, which in turn leads to a leak of calcium through the pump.[37] The resulting high endoplasmic calcium concentration leads to caspase activation, release of apoptotic factors, DNA cleavage by calcium-dependent endonucleases and eventually cell death. This inhibition proceeds with remarkably high specificity, which makes thapsigargin a very useful chemical biology tool for the investigation of calcium efflux. Despite intense medicinal chemistry efforts, its exact binding mode remains unknown,[38] but its potency has led to the development of mipsagargin, a thapsigargin-based prostate-specific membrane antigen (PSMA)-activated prodrug, for the treatment of PSMA-positive solid tumours and currently under clinical investigation.[39] Despite the complexity of this molecule, the Baran group recently demonstrated that it can be accessed synthetically with scalable chemistry.[40]

As further diversification of sesquiterpenes, a number of natural products are the result of the oligomerisation of simpler sesquiterpene lactones.[41] The Lei group has recently been very active in the total synthesis and biologic evaluation of oligomeric sesquiterpene lactones. Ainsliadimer A (Fig. 1E) was thus shown to be a potent inhibitor of the NF-κB pathway, selectively binding to an allosteric pocket of IKKα/β by covalently engaging conserved cysteine 46.[42] Notably, a biotinylated version of the molecule was used in a pull-down assay showing essentially a single band corresponding to IKKα/β as compared to a control molecule wherein the Michael acceptor is reduced. This is remarkable as this is the first allosteric covalent kinase inhibitor. In contrast, Ainsliatrimer A (Fig. 1F) was found to be a highly selective PPARγ antagonist.[43] Although the exact binding mode is unknown, this occurs covalently as demonstrated by knock-down experiments to confirm the nature of the target and pull-down assays, as above, using a biotinylated probe and a negative control with reduced Michael acceptors. Together, these two examples underline the target selectivity that can be achieved with covalent interactions by slightly modifying the structure and thus the pre-organisation of the initial non-covalent complex.

Eudesmanolides are another subset of sesquiterpene lactones. With its 6,6,5 ring system and an α-*exo*-methylene moiety, IJ-5 (Fig. 1G) is the main active ingredient from extracts of the medicinal herb *Inula japonica* used in traditional Chinese medicine for the treatment of inflammatory diseases such as bronchitis, arthritis and hepatitis.[44,45] It was recently reported to inhibit the NF-κB pathway. In order to identify the exact target and mode of action, IJ-5 was grafted onto sepharose beads, and pull-down and proteomic experiments revealed that IJ-5 covalently engages UbcH5c, a key ubiquitin-conjugating enzyme, by reacting specifically with the active site cysteine 85.[46] This enzyme is one of a family of 50 E2 enzymes and is indeed involved in the NF-κB pathway, and IJ-5 was shown to inhibit TNF-α induction of this pathway. IJ-5 was also tested against different protein kinases involved in this pathway, including IKKα/β and found not to be active, despite the redundancy of the α-*exo*-methylene moiety in IJ-5 and ainsliadimer A.

Another prominent subset of sesquiterpene lactones includes the heliangolides. EM23 (Fig. 1H), extracted from *Elephantopus mollis* used in traditional Chinese medicine for various conditions, presented anticancer properties among others. In their investigation on the cytotoxicity mechanism of EM23 against chronic myeloid leukaemia K562 cells and acute myeloid leukaemia HL-60 cells, Liu and coworkers established that it induces caspase-mediated PARP cleavage, DNA breakage and apoptosis. Most importantly, they also noticed that EM23 increased reactive oxygen species levels and pre-treatment with *N*-acetylcysteine reversed EM23-induced apoptosis. They also observed the down-regulation of thioredoxin (Txr) and thioredoxin reductase (TrxR), two enzymes involved in the cellular redox balance. More specifically, TrxR has two redox active residues at its C-terminus, namely cysteine 497 and selenocysteine 498. While time-dependence and mass spectrometry analysis revealed covalent modification of one of those two residues, the pH-dependence confirmed that modification takes place at selenocysteine 498.[47] Suppression of the Trx/TrxR system leads to increased intracellular levels of reactive oxygen species, but also increased phosphorylation of ASK1 and activation of downstream pro-apoptotic MAPK kinases JNK and p38, ultimately leading to apoptosis. Furthermore, the Trx system is also involved in regulation of the NF-κB pathway, and the authors proposed that by suppressing the Trx system, EM23 also effectively blocks the TNFα-induced activation of NF-κB-mediated anti-apoptotic pathways.[48]

3. Beyond the Sesquiterpene Lactones

While sesquiterpene lactones represent a broad and structurally varied source of covalent inhibitors, covalent modifiers can be found in other families of natural products, including higher order terpenoids, polyketides, flavonoids and polyphenols (Fig. 2). In some cases, the covalent inter-

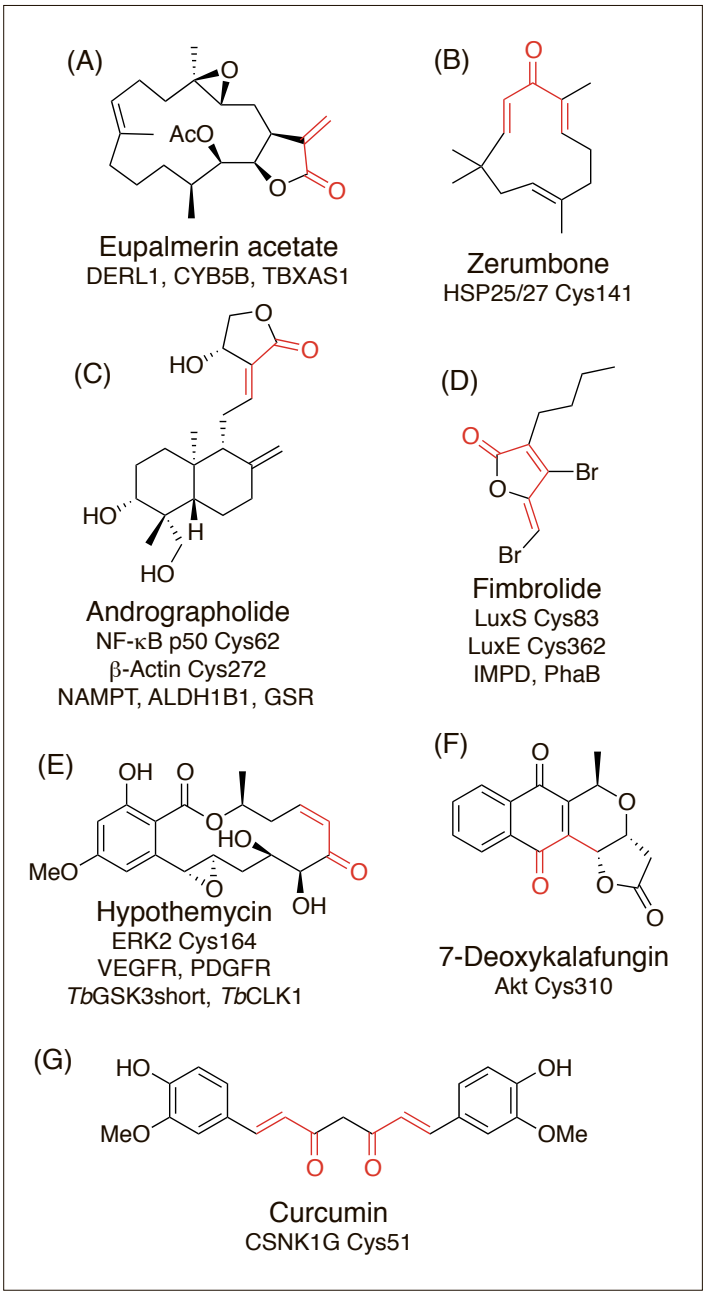


Fig. 2: Selected natural covalent modifiers with their target protein and the modified residue.

action is established but the exact binding site has not been uncovered; these natural products will nonetheless be discussed to highlight a molecular basis for the mechanism resulting in covalent interaction.

Eupalmerin acetate (Fig. 2A) is a cembranoid dipterpene isolated from the Caribbean gorgonian coral *Eunicea succinea*.[49,50] When tested for its cytotoxicity against the human malignant glioma U87-MG and U373-MG cell lines, eupalmerin acetate displayed promising potency. Further studies on the cytotoxicity mechanism revealed that eupalmerin acetate induces apoptosis by translocating apoptosis regulator Bax from the cytoplasm to the mitochondria leading to a loss in mitochondrial membrane potential. Eupalmerin acetate was also found to increase levels of phosphorylated JNK, suggesting the involvement of the JNK pathway in the apoptosis; this was confirmed by the rescue of cells using JNK inhibitor SP600125. In addition, this compound was shown to be active *in vivo*, suppressing the growth of murine malignant glioma xenograft models. The exact targets were however unknown, and a collaboration between the Cravatt and Romo labs led to the synthesis of an alkyne-tagged version of eupalmerin acetate as a probe for proteomic studies.[51] Competitive activity-based protein profiling (ABPP) using stable isotope labelling by amino acids in culture (SILAC) allowed the identification of seven putative targets including DERL1 associated to cancer cell proliferation, CYB5B and TBXAS1 both overexpressed in cancer. Further work using the alkyne-tagged probe should further shed light onto the exact binding mode of eupalmerin acetate for potential clinical applications.

Zerumbone, obtained from the tropical plant *Zingiber zerumbet smith*, is a cyclic sesquiterpene with a cross dienone (Fig. 2B).[52] Biological studies have revealed that it interferes with free radical generation, increases levels of pro-inflammatory proteins, inhibits cancer cell proliferation and induces apoptosis.[53] Zerumbone also alters the oligomerisation of chaperones HSP25/27 involved in apoptotic events, promoting the cross-linking of two HSP25/27. This is supported by the observation that the HSP25 C141A mutant has reduced sensitivity to zerumbone, and the unsaturated ketone is essential for this cross-linking.[54] Early attempts to identify these targets using zerumbone-bound sepharose gel and a zerumbone-biotin conjugates were unsuitable for target validation in live cells. In order to address this issue, Tate and coworkers designed an alkyne-tagged probe based on zerumbone.[55] Using the 'spike-in'-SILAC methodology, they were able to identify 20 significant new covalent targets for zerumbone, several of which are involved in apoptosis. The target proteins responsible for the cytotoxicity and the exact binding site remain however to be demonstrated. This example is however noteworthy in light of the possibility of using cross-conjugated ketones for the cross-linking of proteins, in analogy to disulphide bonds.

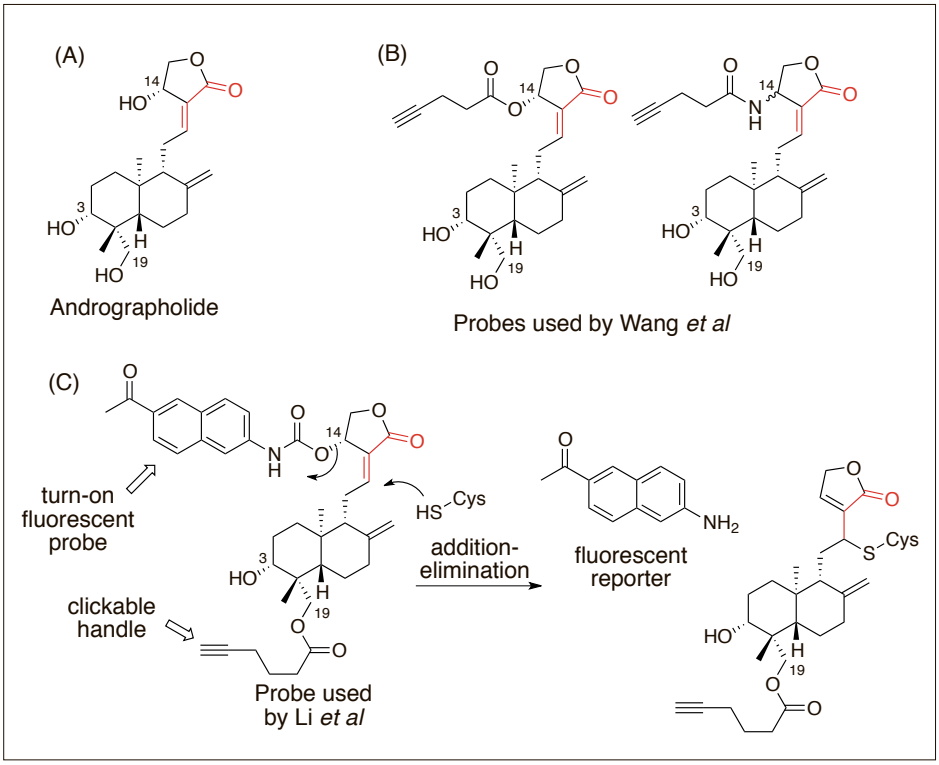
Isolated from the leaves of *Andrographis paniculata* used in traditional Chinese medicine, andrographolide (Fig. 2C) displays a broad range of biological activities, including anti-bacterial, anti-inflammatory, anti-malarial and anticancer to name but a few. Most remarkably, recent studies have shown that it has cytotoxicity against cancer stem cells responsible for multiple myeloma with high selectivity over normal hematopoietic stem cells.[56,57] It is a bicyclic diterpenoid with an α-*exo*-alkylidene-γ-butyrolactone moiety akin to the above-mentioned sesquiterpene lactones. In order to shed light on the origin of these bioactivities, Wang *et al* took advantage of the C-14 alcohol to attach an alkyne tag as an ester.[58] The ester however proved unstable for proteomic profiling, due to an addition-elimination sequence, which precluded efficient labelling (Scheme 1). This was addressed by making an amide instead, and with this probe they were able to identify selective binding at cysteine 62 of NF-κB p50 but also at cysteine 272 of β-actin. Independently, Li *et al* elegantly took advantage of the addition-elimination sequence by designing a bifunctional probe with an alkyne tag at the C-19 alcohol and a turn-on fluorescent reporter attached at the C-14 alcohol.[59] While the alkyne tag serves as a clickable handle for proteomics, the fluorescent reporter is released upon addition of a cysteine residue

for fluorescence imaging. With this probe in hand, they confirmed that andrographolide targets NF-κB p50 and six additional targets, notably including NAMPT, an enzyme involved in NAD biosynthesis, as well as ALDH1B1 and GSR, both proteins being involved in balancing reactive oxygen species. They however also warn that andrographolide is quite promiscuous and rapidly deactivated by glutathione. Nonetheless, its molecular mechanism of action, *i.e.* the addition-elimination sequence, is remarkable and should inspire the development of new sensors for covalent inhibitors as well as targeted delivery methods.

Isolated from the marine algae *Delisea pulchra*, with their polybromide-substituted butenolide, the fimbrolide natural products have very unusual structures (Fig. 2D).[60] Previous studies had shown that they potently disrupted quorum sensing of the marine bioluminescent gram-negative *Vibrio* bacteria, but they are also effective against other pathogenic bacteria. The bromoolefin moieties are very well-poised to undergo an addition-elimination sequence (Scheme 2) for the formation of covalent adducts with nucleophilic residues in proteins. *In vitro* experiments showed that they do indeed covalently modify LuxS leading to its inhibition and the covalent adduct was proposed to be at the non-catalytic cysteine 128 in LuxS; the proteomic target profiling had however not been carried out. In order to fill this gap, Sieber and coworkers prepared a range of alkyne-tagged fimbrolide analogues and used them for competitive activity-based protein profiling (ABPP) with quantitative mass spectrometric analysis.[61] This allowed them to confirm that LuxS is indeed a covalent target and that binding takes place at catalytic cysteine 83 instead of cysteine 128 as previously proposed. They additionally identified LuxE as another major target, which is covalently modified at catalytic cysteine 362. Both enzymes are essential for bioluminescence and quorum sensing. Two additional targets were identified, namely the hitherto uncharacterized CBS domain protein inosine monophosphate dehydrogenase related protein (IMPD) and PhaB, involved in polyhydroxybutyrate biosynthesis. Besides the discovery of quorum sensing modulators, the addition-elimination sequence represents a very appealing addition mechanism as it precludes potential reversibility in the conjugate addition event.

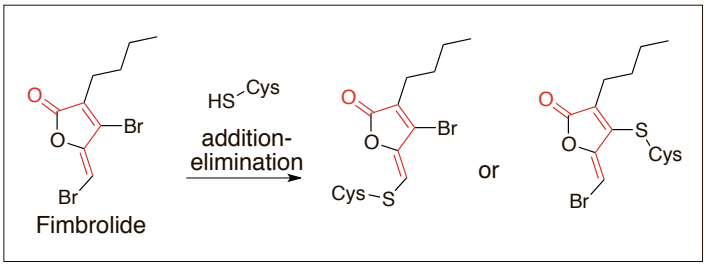
The resorcylic acid lactones are a broad family of polyketide natural products produced by different fungal strains and have shown promise as potent ATP-competitive kinase inhibitors.[62] Indeed, their resorcylic acid moiety is able to recapitulate the essential hydrogen-bonding network involved in the binding of ATP's purine to the nucleotide binding site of kinases. Hypothemycin (Fig. 2E), which bears a *cis*-enone moiety embedded in its macrocycle, was shown to selectively engage a subset of kinases with a common cysteine residue[63] with selectivity for ERK2's cysteine 164.[64] We and others have studied resorcylic acid lactones related to radicicol, a non-covalent HSP90 inhibitor despite the presence of an electrophilic moiety[65–67] and hypothemycin-related resorcylic acid lactones,[68–70] leading to the discovery of VEGFR and PDGFR inhibitors effective *in vivo*.[71] More recently, hypothemycin was shown by Taunton and coworkers to be efficacious in the treatment of sleeping sickness induced by *Trypanosoma brucei*.[72] Quantitative proteomics revealed that it covalently inactivates kinases with a CDXG motif, including *Tb*GSK3short and *Tb*CLK1, which was previously uncharacterized but is responsible for the toxicity of hypothemycin towards *Trypanosoma brucei*, which was efficiently killed *in vitro* and in infected mice.

The pyranonaphthoquinone lactone natural products include 7-deoxykalafungin (Fig. 2F), lactoquinomycin and frenolicin B, which have all been demonstrated to selectively and potently inhibit Akt, a kinase often hyperactivated in cancer, diabetes and neurodegenerative diseases, with IC_{50} values of 150–200 nM. They covalently engage the conserved cysteine 310 in apoptosis suppressor Akt with high selectivity over other enzymes of the AGC family, over half of the members of which contain this cysteine.[73] The structure does however not lend itself to easily predict the mechanism of binding of the pyranonaphthoquinone lactones. Their activation as Michael acceptors was traced to an *in situ* thiol-based reduction of the quinone moiety with concomitant elimination of the lactone, resulting in the formation of a quinone methide, a potent Michael acceptor (Scheme 3).[74] Ellis and coworkers undertook to examine the structure–activity relationship of 7-deoxykalafungin towards making simpler analogues. While their work confirmed the necessity of the



Scheme 1. (A) Andrographolide; (B) Probe used by Wang *et al*.;[58] (C) Bifunctional probe used by Li *et al*.[59]

Scheme 2. Covalent attachment of fimbrolide to cysteine residues.



quinone and lactone moieties for inhibition of Akt, they also found that when the pyran ring is removed, another PKAα could be targeted covalently at cysteine 199. The activation mechanism is particularly interesting and inspiring for the design of probes and drugs with masked Michael acceptors.

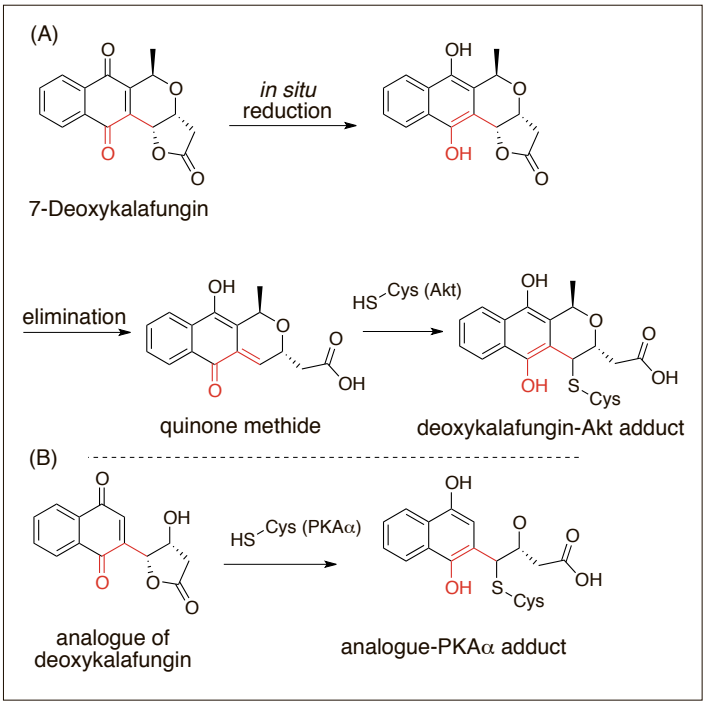
Isolated from turmeric *Curcumin longa*, curcumin (Fig. 2G) has widely been used as a spice but also has anticancer and anti-inflammatory properties.[75] Consumed as a spice, it is obviously well tolerated in high dosage by humans and owing to its pharmacological properties, it has been investigated in clinical trials for the treatment of multiple myeloma, colon and pancreatic cancer. Curcumin has a particularly interesting structure: it is a diarylheptanoid with a β-diketone, both α,β-unsaturated, which suggests that it may be able to engage in covalent interactions with nucleophilic amino-acid residues and in particular cysteines. Although early observations linked curcumin to decreased phosphorylation of Akt, little is known about the direct targets of curcumin. In order to address this question and as part of a study on ethynyl benziodoxolone (EBX) reagents as new cysteine labelling reagents, Adibekian and Waser engaged in competitive activity-based protein profiling using both EBX and an alkyne-tagged version of curcumin.[76] This allowed them to identify several target proteins, including casein kinase I gamma (CSNK1G) at cysteine 51 in the P loop in the vicinity of the ATP-binding site of the kinase. Since CSNK1G is directly involved in the Akt signalling pathway, this may explain the decreased Akt phosphorylation levels and cytotoxicity. Independently, Carroll and coworkers are actively involved in the study of the oxidation of cysteines to their sulfenic acid counterpart in signalling events in analogy to phosphorylation events.[77] They recently showed on a model polypeptide that curcumin could also play the role of a nucleophile thanks to its β-diketone moiety (Scheme 4), which further expands its potential as a covalent inhibitor.

4. Other Modes of Action

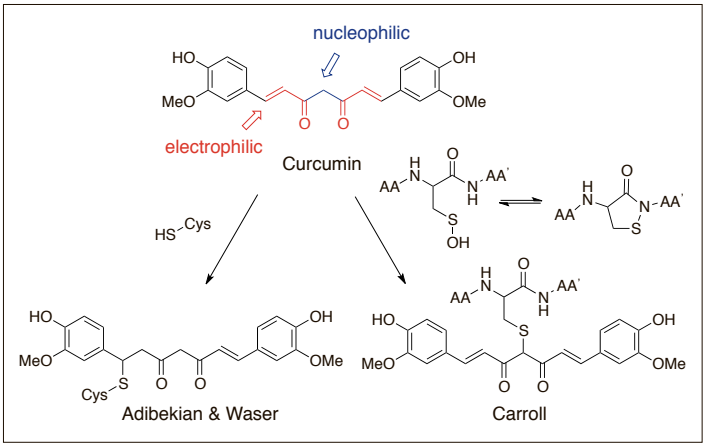
This review has so far discussed the importance of Michael acceptors as selective covalent modifiers of cysteine residues in their target proteins. A range of other natural products also covalently engage cysteine residues with sometimes rather uncommon activation mechanisms in order to unmask the reactive Michael acceptor involved in cysteine modification. From this point of view, the literature on natural products is rich with unusual activation mechanisms responsible for their high cytotoxicity in the low nanomolar to picomolar range and they have provided great inspiration for the development of drugs currently being investigated for targeted therapy. For example, the calicheamycins are extremely potent cytotoxins: they bind to the minor groove of DNA and potently induce DNA damage and strand scission upon disulphide bond cleavage, intramolecular *thia*-Michael addition and concomitant Bergman cyclisation.[78] The duocarmycins are another family of extremely potent toxins with a strained cyclopropane that acts as the warhead in DNA-alkylation chemistry.[79] This feature has been harnessed in the design of prodrugs, wherein the cyclopropane is formed in response to the unmasking of a phenol.[80] In this part novel warheads recently identified which display unusual reactivity towards biomolecules will be described.

The human microbiome is receiving increased attention owing to the interplay with its ecology and human health. Some strains of *Escherichia coli* are responsible for inflammation-induced colorectal cancers due to DNA double strand breaks,[81] and small molecules were predicted as mediators in the inflammation process but not characterized.[82] Independently, based on the study of the colibactin gene cluster, three groups reported the putative colibactin warhead as well as its activation towards DNA alkylation (Scheme 5).[83–85] Briefly, upon cleavage of the asparagine residue, the resulting amine intramolecularly cyclizes to an iminium, which in turn enhances the electrophilicity of the cyclopropane trigger and alkylates DNA in a vinylogous homo-Michael addition. This is very much in analogy with the duocarmycin cyclopropane-trigger compounds, both from the prodrug and alkylation point of view, which should inspire the development of novel activation modes for targeted drug delivery.

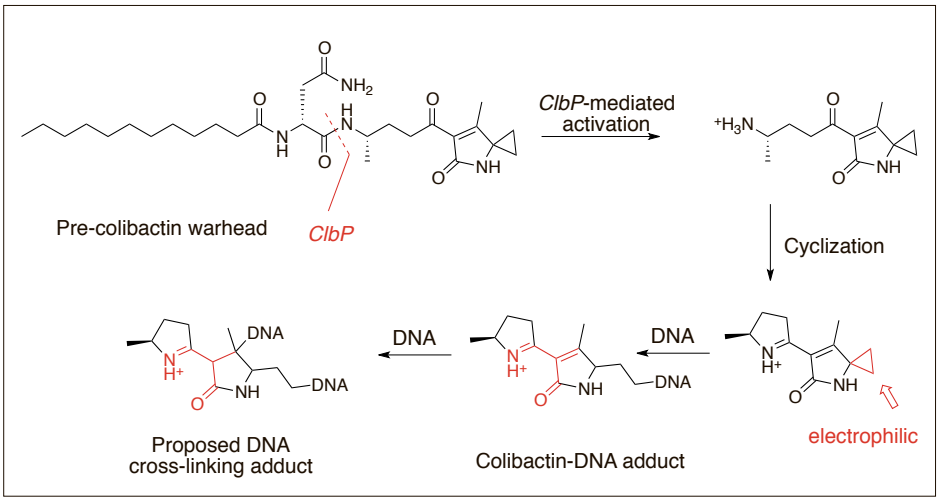
Isolated from fungi of the *Bipolaris genus*, ophiobolin A is a fungal phytotoxin made by the fungus to attack plant



Scheme 3. (A) 7-Deoxykalafungin activation and trapping of Cys310 (Akt); (B) Simplified analogue targets PKAα.

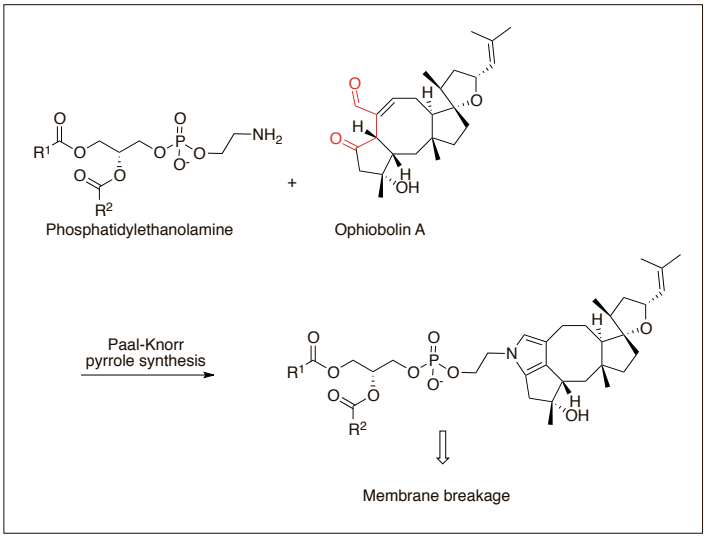


Scheme 4. Curcumin as an electrophile for cysteine residues or as a nucleophile for cysteines oxidized to the sulfenic acid.



Scheme 5. Activation towards the colibactin warhead and proposed DNA cross-linking mechanism.

Scheme 6. Ophiobolin A.

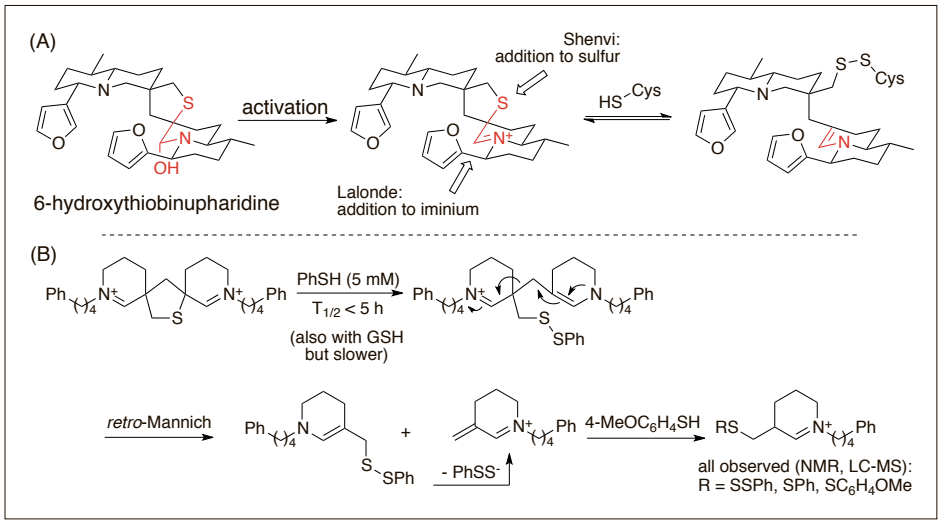


cells (Scheme 6). It is cytotoxic due to parapoptosis at nanomolar concentrations against a range of cancer cell lines, including glioblastoma cells, which translated into anti-tumour activity in mice models.[86] Structurally, it possesses a 1,4-dicarbonyl moiety necessary for bioactivity and which undergoes Paal-Knorr pyrrole synthesis with primary amines, leading to the proposal that ophiobolin A may covalently modify lysine residues in its hitherto unknown target protein.[87] In order to determine the mode of action, O'Shea and coworkers generated loss-of-function mutants of the KBM7 cell line for screening a loss of effect of ophiobolin A, and found that inactivating the pathway for the formation of phosphatidylethanolamine indeed led to a loss of response.[88] They could finally identify the target of ophiobolin A as phosphatidylethanolamine itself. The resulting covalent adduct was shown to disrupt model membranes, which suggests that the cytotoxicity is due to membrane breakage in cancer cells. With an increasing interest for covalent modifiers of lysine side chains in proteins, these results are especially important for the design of novel warheads and will certainly find applications in drug design.

The *nuphar* dimers represent a small subset of terpene alkaloids, of which four series are described depending on the stereocentres at the thiaspirane linkage between the 'monomers' (Scheme 7). They possess a variety of biological activities including immunosuppressive, antimicrobial, and anticancer by inducing apoptosis. More interestingly, *in vitro* assays for metastasis indicated low nanomolar efficacy for the most potent member 6-hydroxythiobinupharidine, as well as in murine lung cancer models.[89] The exact mode of action of those compounds is not well understood but a structure–activity relationship study suggests that the hemiaminal moiety is required and it needs to be adjacent to the carbon–sulphur bond in the thiaspirane. While the necessity for a hemiaminal points to the formation of an iminium species, which could in turn behave as an electrophile for nucleophilic residues, the reason for its location with respect to the thiaspirane ring remained to be understood.[90] Furthermore, replacing the sulphur atom by an oxygen atom also leads to complete loss of activity. These observations led Shenvi and Cravatt to hypothesize that the sulphur atom may be the actual electrophilic moiety.[91] In a series of FT-IR and NMR experiments with warhead models and thiols, they were able to show that the thiol does indeed rapidly but reversibly react at the thioether linkage, with concomitant formation of an enamine, but also that this product in dihydroxy derivatives can undergo retro-dimerization to afford a highly activated α,β-unsaturated electrophile, which in turn serves as a trap for further thiol nucleophiles (Scheme 7B). Furthermore, using smaller analogues and treatment with an iodoacetamide-alkyne probe in the isotopic tandem orthogonal proteolysis-activity-based protein profiling isoTOP-ABPP, they were able to modify cysteines in Jurkat cell lysates. Unfortunately, no target was formally identified, but these results open possibilities for the formal identification of covalent targets. Most importantly, the hemiaminal thiaspirane motif is unprecedented and understanding the mechanism at the molecular level can allow the incorporation of this motif for the design of novel prodrugs and covalent inhibitors.

5. Conclusion and Outlook

Natural products often contain mildly reactive functionalities that can engage a target through covalent interactions. The propensity of such functionalities across different classes of natural products attests to the effectiveness of this inhibition modality. The resurging interest in covalent inhibitors as therapeutics brings renewed attention to natural products as leads and a source of inspiration. Progress in chemical proteomics enables a better understanding of the selectivity of covalent modifiers, which is essential to overcome the prejudices of promiscuity and toxicity. The sesquiterpene lactone family is a very rich source of covalent inhibitors. Often, these natural products are extracted from plants with a historical use in traditional medicine, offering preliminary toxicology assessment in human. Despite the redundancy of the α-*exo*-methylene-γ-butyrolactone moiety in sesquiterpene lactones, diverse target selectivity is achieved owing to the structural diversity of molecules this functionality is imbedded into. With thousands of members in this family, it is tempting to extrapolate that more covalent modifiers with different targets and binding modes will be identified in the future and that other natural product families will continue to



Scheme 7. (A) 6-Hydroxythiobinupharidine and proposed reactivity towards nucleophilic thiols; (B) Model compounds and proposed thiol-induced retro-dimerization and thiol capture.

contribute novel covalent modifiers. The identification of masked covalent modifiers amongst natural products has already inspired the design and development of prodrugs. The recent discovery of yet new pro-electrophilic moieties illustrates that there is a diverse warhead chemical space beyond traditional Michael acceptors.

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