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1330 *Mini-Reviews in Medicinal Chemistry*, 2012, *12*, 1330-1344

Reversible Michael Additions: Covalent Inhibitors and Prodrugs

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Abstract: Covalent inhibition is an efficient molecular mechanism for inhibiting enzymes or modulating the function of proteins and is found in the action of many drugs and biologically active natural products. However, it is has been less appreciated that the formation of covalent bonds can be reversible or irreversible. This review focuses on biologically active compounds that are Michael acceptors and how the reversible nature of the Michael addition reaction influences biological activity and how this can be exploited in designing prodrugs.

Keywords: Covalent inhibition, Michael addition, natural products, prodrug, reversible.

1. INTRODUCTION

One of the dominating concepts in pharmaceutical research and drug discovery is the concept of "one drug-one target" that builds on the idea of a highly selective molecule binding in a well-defined manner to a single biological target. This binding event should then elicit the desired pharmacological response and affect the disease state in a desired way. This concept most often refers to small organic molecules that bind in a reversible, non-covalent, manner to a well-defined binding pocket of the target protein. However, this concept is not always reflected by reality. Many approved drugs bind to and modulate multiple targets and that this polypharmacology is actually necessary for their clinical efficacy. This is especially true for many CNS drugs. But this non-selectivity has often been discovered after the fact and designing a desired polypharmacologic profile is still considered highly challenging. Another class of drugs that fall outside this concept is covalently binding drugs.

The existence of reactive, electrophilic functionalities in drugs has long been viewed as a liability and a risk for toxic events, including immunogenicity caused by protein-inhibitor adducts, and have thus been avoided by medicinal chemists and shunned by toxicologists. The use of a highly reactive and irreversible covalent binding compound as a drug, could lead to idiosyncratic toxicities caused by haptenization of target proteins leading to the activation of the immune system caused by the generation of antibodies that will recognize the covalently modified endogenous protein as "foreign". In addition drugs can also be metabolized to give electrophilic and reactive metabolites, which in turn can cause irreversible toxicities. To avoid such issues, the pharmaceutical industry has developed screens to identify and eliminate potentially reactive compounds and drug metabolites [1].

Irreversible inhibition or covalent modification of drug targets has therefore not been considered as a tractable means of achieving a safe pharmacological response. However, upon closer examination this mode of action is relatively common among approved drugs (e.g. omeprazole, clopidogrel, orlistat, β-lactam antibiotics) [2]. In fact 30% of the approved drugs that act on enzymes are irreversible inhibitors although many of these were discovered serendipitously and their exact molecular mechanism of action was often only elucidated afterwards. Nature however, has often capitalized upon this molecular mode of action to achieve biological activity [3]. Numerous natural products contain electrophilic moieties and react covalently with nucleophilic functional groups in their target molecules. The formation of a covalent bond between a biologically active compound and its target protein can be either reversible or irreversible and the covalent bond formation can arise from several different chemical reactions; alkylation, hemiketal formation, acylation, metal binding or complexation, disulfidebond formation, 1,4-conjugate addition (the Michael addition) and the reaction between a nitrile and a hydroxyl group (a Pinner reaction).

One major concern with using electrophilic drugs is that the reactivity will outweigh and preclude any selectivity, causing toxic insult due to covalent modification of biomolecules, which could lead to initiation of cell damage response and that electrophilic compounds therefore are not optimizable as drug candidates. Proteomic methods can be used as an aid in developing covalent inhibitors where non-selective or promiscuous binders can be discriminated from more selective inhibitors. This includes the use of proteome wide analysis using activity based protein profiling (APBB) [4].

However, with an increase in focus on the molecular mode of action of drugs [5] and the importance of tight binding with slow K_{off} rates [6] or even preferably semi-irreversible or fully irreversible modes of action [7], there has been an increased interest in developing covalently binding drugs. It is believed that covalent inhibition can provide higher biochemical efficiency in target modulation [8]. The possibility of better inhibition kinetics and counteracting resistance has seen a renewed interest in drugs binding in this fashion. Drug residency time and slow off

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1875-5607/12 $58.00+.00

kinetics has been seen as key factors for the success of a potential small molecule drug [4] so methods of optimizing these factors are crucial. Also covalent bonding can be seen as a way of achieving high potency, i.e. sub picomolar potencies, which is not obtainable with reversible non-covalent inhibitors [9]. Results from safety and toxicological studies and clinical trials of novel covalently acting drugs have given increased confidence that this can be a safe and effective approach to developing drugs. Two major strategies can be defined to develop safe and efficient covalently acting drugs.

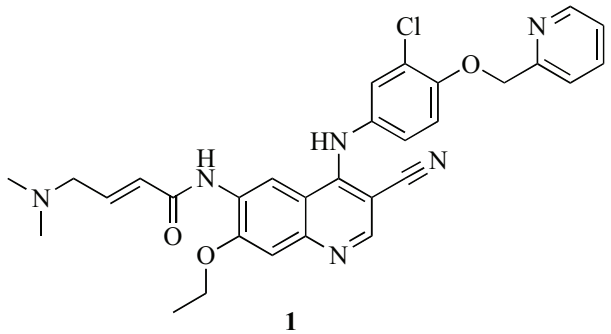

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Fig. (1). Irreversible kinase inhibitor Neratinib (HKI-272).

The first is to incorporate less reactive electrophilic functional groups in the compound, where the formation of a covalent bond is a secondary and final interaction with the target. Here focus is on designing molecules that have a tight optimized binding to the target through reversible interactions and only when the electrophilic moiety is in close proximity to a nucleophilic residue in the target-binding pocket will a covalent bond be formed. This has been termed targeted covalent inhibition (TCI) [10]. This has most successfully been employed in the development of irreversible kinase inhibitors (Fig. **1**) [11]. Here structure based drug design using protein x-ray structures and bioinformatics have helped to identify critical proximal cysteine residues or other nucleophilic groups that can be covalently modified through Michael addition reactions. This irreversible inhibition can lead to improved pharmacodynamic properties, decreased occurrence of resistance and increased selectivity versus other kinases [12]. Several irreversible kinase inhibitors containing reactive Michael acceptor functionalities, e.g. canertinib (CI-1033) [13], pelitinib (EKB-565) [14], neratinib (**1**, HKI-272) [15], afatinib (BIBW 2992, Tovok) [16], are currently being evaluated in both early and late stage clinical trials for various cancers. Interestingly, in the development of pelitinib (EKB-565) it was discovered that 4-dialkylaminobut-2-enamides functioned as superior Michael acceptors as it is believed that the dialkylamino group proximal to the electrophilic double bond functions as an internal base thus promoting the Michael addition by deprotonating the attacking thiol group. Significant work has been done looking into the reactivity of the cysteine reactive moiety of this class of inhibitors in order to determine the balance between reactivity and potency and how the reactive functionality can be expanded beyond Michael acceptors [17]. In another strategy to modify the reactivity of the cysteine reactive warhead functionality of irreversible kinase inhibitors, 3-aminopropanamides that can be converted intracellularly to acrylamides have been prepared [18]. This process can be a metabolically activated process but due the generality of the effects of the 3-aminopropanamides it is more likely to be a spontaneous retro-Michael addition/β-elimination reaction.

Clinical reports from phase II studies are now becoming available for irreversible tyrosine kinase inhibitors and although this novel class of kinase inhibitors does display biological activity, they have not yet demonstrated an increase in overall survival in the tested settings as single agents [19].

The second approach to designing covalent inhibitors, instead depends on more or less highly reactive electrophilic groups where the specific non-covalent interactions with the target are less obvious has been less systematically studied, but that approach can nonetheless give compounds with adequate selectivity and acceptable safety properties. This class of compounds, their mechanism of action and how they can be safe and selective despite high reactivity, are less well understood and have been less studied but a reoccurring feature seems to be that they undergo reversible covalent bond formation.

Reversible covalent inhibitors as protease inhibitors have been extensively studied. This class of inhibitors often contains an aldehyde as an electrophilic functional group that can undergo a reversible formation of a hemiacetal with nucleophilic residues, serine or cysteine, in the active site of the protein. Though the formation of the covalent bond is reversible it can increase the binding affinity up to three orders of magnitude [20]. Aldehydes are not the only electrophilic functional group to participate in reversible covalent bond formation in protease inhibitors. The boronic acid functionality in the proteasome inhibitor bortezomib undergoes reversible boronic ester transesterification with its target thus forming a covalent bond [21]. Also nitriles can be found as reactive electrophilic moieties in protease inhibitors that can undergo reversible covalent bond formation [22].

One of the more common reactive electrophilic functionalities is the α,β-unsaturated carbonyl system, more commonly known as a Michael acceptor. These can undergo addition reactions with carbon, nitrogen, sulphur, and oxygen-based nucleophiles. The two major targets or pathways of highly reactive Michael acceptors are the NF-κB signalling pathway [23] and the Keap-Nrf2 pathway [24, 25].

To enable the design of Michael acceptors as potential drugs, attention has been on modulating the reactivity of the Michael acceptor, i.e. increasing or decreasing the reactivity by changing the electron density of the conjugated double bond [26] E_{LUMO} values of the conjugated double bond have been found to correlate with the biological activity of naturally occurring and synthetic plant phenylpropenoids as well as for cyclopentone prostaglandins. Thus the reactivity of the Michael acceptor, i.e. a small difference between the E_{LUMO} of the electrophile and the E_{HOMO} of the nucleophile, has been seen as the sole determinant of reactivity and risk

of side-effects and less attention has been paid to the dynamics and reversibility of the Michael addition reaction between the electrophile and biological nucleophile (often thiols).

The reversibility of the Michael addition and the reversible formation of covalent bonds between Michael acceptors and biological nucleophiles and how this influences the biological activity of Michael acceptors is the focus of this review.

2. THE MICHAEL ADDITION REACTION

One of the most important electrophilic functionalities in biologically active compounds is the Michael acceptor. The conjugation of a double bond with an electron withdrawing group polarizes the double bond, rendering the β position of the double bond electron deficient and thereby susceptible to nucleophilic attack. This type of reactive functionality is called a Michael acceptor and the addition of nucleophiles to the double bond is called a Michael addition. Michael acceptors are soft electrophiles and preferably undergo addition with soft nucleophiles, e.g. thiols, making them more or less selective for certain biological nucleophiles, i.e. they react more readily with sulfur containing amino acids like cysteine than with nitrogen or oxygen based nucleophilic amino acids like lysine or serine.

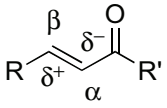


Fig. (2). A Michael acceptor.

The first step of the Michael addition reaction is the deprotonation of the nucleophile with a base followed by nucleophilic attack on the β-position of the polarized double bond (Scheme **1**). Protonation of the intermediate enolate and subsequent keto-enol tautomerization affords the adduct. However, all the steps in the Michael addition reaction are potentially reversible; that is that the nucleophile can be eliminated, thus regenerating the Michael acceptor functionality.

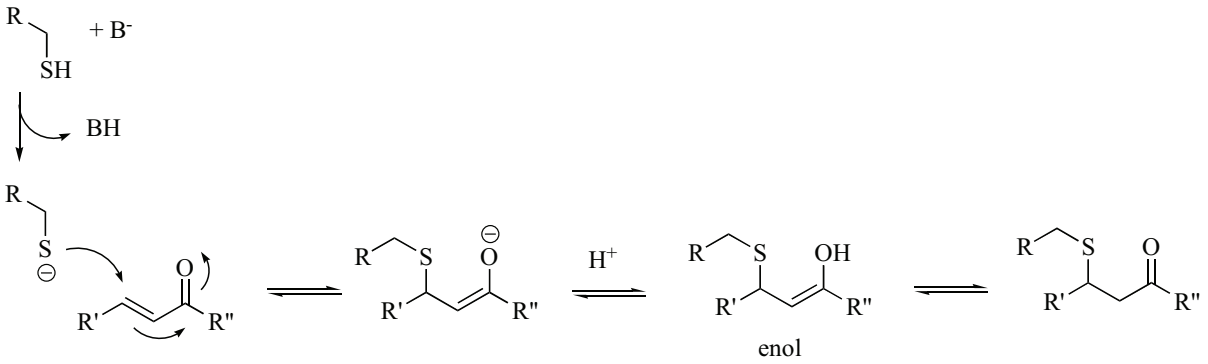
The factors governing the extent of the reversible Michael addition and the position of the equilibrium between free electrophile and nucleophile and the covalently bound adduct have not been fully determined.

The reversibility of the Michael reaction has even been demonstrated in systems where the addition of a nucleophile releases strain in the system but which nonetheless can undergo reversible addition. This was observed with the diterpenoid briareolate ester L [27].

2.1. The Reversibility of the Michael Addition

Many studies have been aimed at studying the kinetics and factors influencing the addition of thiols to Michael acceptors, mainly α,β-unsaturated ketones, but less emphasis has been placed on studying the factors that determine the reversibility of the reaction and the factors influencing the stability of the thiol-Michael acceptor adduct. These factors should be of importance to the biological effects and toxicological properties of thiol reactive Michael acceptors. The reversibility should depend on the energy barrier for the reverse of the rate-determining step and should affect the net-reactivity of the addition.

Early studies to correlate the biological activity and toxicity of α,β-unsaturated carbonyl compounds, acrolein in particular, used the addition of glutathione as a model reaction for the addition of reactive Michael acceptors to protein thiol groups. Glutathione (GSH) is also one of the most abundant soft nucleophiles in cells and is present in high concentrations (0.5-10 mM) [28]. Glutathione plays important roles in cells in maintaining cellular redox levels and detoxifying processes and glutathione can act as both nucleophile and reducing agent. It can either react spontaneously with electrophiles or be conjugated to them through the action of glutathione transferase (GST) enzymes. Excessive lowering of free glutathione levels in healthy cells could trigger excess ROS generation and induce cellular toxicity. Therefore it is essential that the binding of free reduced glutathione is reversible to prevent decreasing the glutathione levels to detrimentally low levels and also to avoid deactivation of the drug by forming irreversible glutathione adducts that can be transported out of cells. However, lowering GSH levels in the target cells could be a productive treatment strategy for increasing the susceptibility these cells towards other treatments as GSH is part of the protective and drug resistance mechanism of cells. This could be a method of sensitizing cancer cells towards chemotherapy. Glutathione conjugation is not straightforward



Scheme (1). The Michael addition reaction.

as it is often a reversible process and the fate of glutathione conjugate can either mediate or abrogate the effect of short-lived and reactive electrophiles [29, 30].

In the model study using glutathione, the known biological activity of the α,β-unsaturated carbonyl compounds correlated very well with a fast addition reaction with glutathione and low reversibility, i.e. the formation of stable adducts [31]. The factors affecting the forward addition reaction correlated well with the electron withdrawing effects and steric hindrance of the Michael acceptor but the properties affecting the stability and reversal of the adducts were less obvious. In some cases stable cyclic hemiactals are formed. There was a large variation in reaction rates depending on the structure Michael acceptor, showing that reactivity of Michael acceptors can vary greatly. This variation can be over five orders of magnitude and the general trend in reactivity is; aldehyde>ketone>ester>amide> carboxylate. As expected, the addition reaction is highly pH dependent with increased reaction rates at higher pH. This should be taken into account when considering the reactivity of Michael acceptors in different tissue, cellular compartments and even within proteins where the local conditions can vary greatly due to desolvation effects and intra-protein interactions. The pKa of the thiol SH group can change considerably depending on the environment e.g. surroundings in a protein and this will have an effect on the thermodynamics and reversibility; the pKa of the attacking thiol will affect the rate of both the forward and the reverse reaction. The equilibrium of the Michael reaction will depend on the acidity of the thiol and the acidity of the enol form of the adduct (Table **1**).

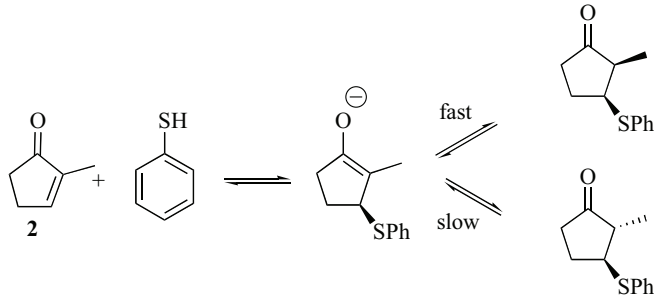
Table 1. The pKa Values of Different Thiols [32]

Thiol	**pKa**
Glutathione (GSH)	8.7, 8.8
Dithiothreitol (DTT)	9.2, 10.1
Mercaptoethanol	9.6
Cysteine	8.3
Cysteine-Me ester	6.7
N-acetyl cysteine	9.5
Cystamine	8.6, 9.0

Utilizing computational transition-state models of the Michael addition reaction have confirmed the importance of the reversibility of the Michael addition in predicting the reactivity of α,β-unsaturated compounds [33]. Interestingly, these calculations showed that the intermediately formed enol of the adduct, is the key reactive intermediate and that the reverse reaction takes place from the enol form and not through direct elimination of the nucleophile from the final product. Determining the keto-enol equilibrium could be a good predictor of the rate and extent of the reverse reaction and factors stabilizing either the keto or enol form will influence the rate of the reverse reaction and the equilibrium.

This has partially been demonstrated in a study of the stability of Michael adducts formed from β-morpholinoethanethiol and α,β-unsaturated carbonyl compounds. The thermal reversibility was shown to greatly depend on the pKa of the hydrogen in the α-position of the formed adduct. Adducts of methylenemalonic diesters were significantly more readily cleaved compared to the corresponding mono esters [34].

In studies of the base catalyzed addition of benzene thiol to 2-methyl cyclopentenone (**2**) it was discovered that the apparent quantitative addition was reversible and that an equilibrium existed (Scheme **2**) [35]. The reverse reaction could be reversed by the addition of a metal-salt. There was also an equilibrium between the cis and trans addition products indicating the formation of the corresponding enol or enolate-base pair allowing the formation of the more stable trans product over time.



Scheme (2). Dynamic and reversible addition of thiophenol to 2-methyl cyclopentenone.

The kinetics and reversibility of the Michael reaction are not only dependent on the structure of the Michael acceptor and the attacking nucleophile but they are also highly sensitive to the reaction conditions where the polarity and pH of the solvent play large roles where the reaction is reversible in protic solvents.

Reactive electrophiles, including Michael acceptors, have been extensively studied due to their chemical toxicity and ability to cause cellular damage leading to skin sensitization [36]. These compounds are often reactive electrophilic metabolites of hazardous chemicals or electrophilic metabolites of endogenous compounds produced through oxidative stress. The toxicity and ability of electrophiles to cause cellular damage is linked to their ability to covalently modify cellular macromolecules. Particularly susceptible to covalent electrophilic and oxidative modifications are highly nucleophilic cysteine thiols. Cellular sensors of damage and oxidative stress often rely on covalent thiol modifications for detecting cellular toxicity and for initiating signalling and adaptive responses to electrophiles. But a recent study has indicated that the response and toxicity caused by thiol modifying electrophiles is highly dependent on the nature of the electrophile and the stability of the protein-thiol adduct [37].

Comparing the thiol reactive protein labeling electrophiles *N*-iodoacetyl-*N*-biotinylhexylenediamine (**3**, IAB) and 1-biotinamido-4-(4'-[maleimidoethylcyclohexane]-carboxamido) butane (**4**, BMCC) showed that not only was the protein

labeling profiles different, but the probes also showed different cellular effects. Despite that both electrophiles react readily with thiols, only **3** displayed cytotoxicity towards HEK293 cells. The major difference between the **3** and **4** is that **3** forms irreversible protein adducts in contrast to the BMCC adducts which not stable under cellular conditions. Presumably the maleimide moiety undergoes hydrolysis (possibly catalyzed by an imidohydrolase) followed by a retro-Michael addition liberating the protein thiol. Thus a transient and reversible adduct formation will not elicit a strong cellular response through activation of damage-signaling pathways and the dynamic nature of the protein-electrophile covalent adduct formation must be taken into consideration when looking at cellular responses to various electrophiles and possible toxic effects.

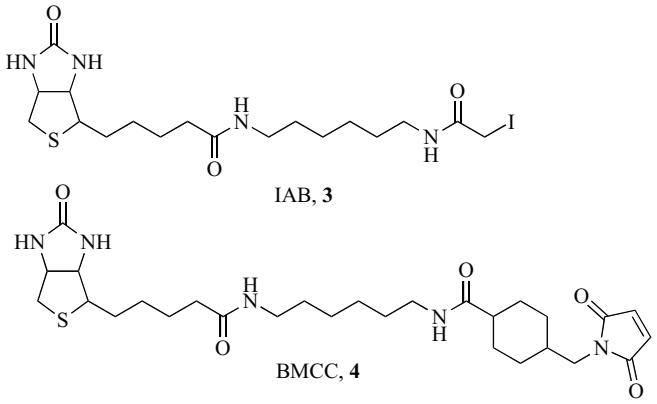


Fig. (3). Thiol reactive labelling reagents.

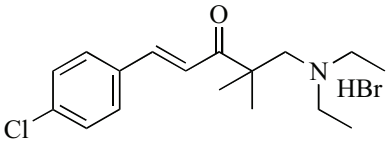


Fig. (4). CDDP.

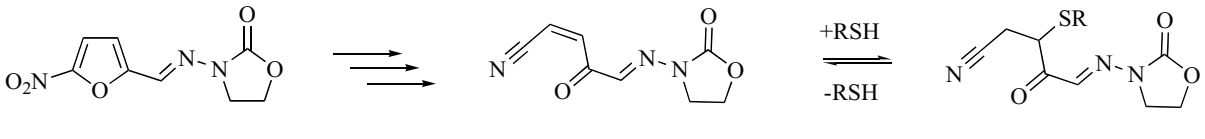
But all cellular thiols do not react equally with Michael acceptors. There is a discrepancy between the reversibility of the addition of low molecular weight thiols to Michael acceptors and the seemingly irreversible binding to thiol groups of macromolecules. This was first studied using the α,β-unsaturated ketone CDDP (1-*p*-chlorophenyl-4,4-dimethyl-5-diethylamino-1-penten-3-one hydrobromide) [38]. CDDP does not react with N- and O-nucleophiles but readily reacts with the thiol of cysteine, glutathione and mercaptoethanol. Observing the UV-spectra of CDDP and using an irreversible thiol reacting reagent (2-chloro-1,3-dinitrobenzene, CDNB) in competitive experiments, it was determined that the addition of low molecular weight thiols such as cysteine and glutathione to CDDP was reversible under physiological conditions but the binding to exposed thiols in cysteine containing proteins was apparently irreversible. The authors speculate that restricted movement of CDDP when covalently bound to the protein prevents the enol tautomer of the CDDP-protein thiol adduct to obtain the proper orientation for elimination of the thiol and regeneration of the conjugated double bond of the Michael acceptor CDDP. This is similar to the argument for the lack of reversibility of unsaturated cyclopentenone prostaglandins bound to thiols in macromolecules (*vide infra*).

If the irreversibility of Michael acceptors to thiols present in proteins and macromolecules is a common feature then to achieve necessary selectivity and safety of small molecule inhibitors containing reactive Michael acceptor functionality other factors in the design of such compounds must be taken into account. This could include attenuating the reactivity, modulating the physical chemical properties (e.g. logP) or increasing the non-covalent interactions of the drug to the intended target(s). But the formation of irreversible covalent bonds between reactive Michael acceptors and thiol containing proteins is not a general phenomenon.

The veterinary antbiotic furazolidone **5** is converted to a reactive acrylonitrile metabolite by swine liver microsomes and this electrophilic metabolite can undergo reversible Michael addition reactions with thiols (Scheme **3**) [39]. This metabolite also forms covalent adducts when incubated with microsomal proteins. However, these covalent adducts are not stable and the covalent bond formation is reversible; when incubated with excess mercaptoethanol the reactive metabolite is released from the protein as witnessed by the formation of the corresponding mercaptoethanol adduct. Moreover this reversibility shows a strong pH dependence with an optimal reversal between pH 7 and 10.

2.2. The Biological Relevance of Reversible Michael Additions

The occurrence of reversible Michael additions has been implicated in many biological processes and as the source of activity of biologically active compounds, including the transient activity of the pyrethroid insecticide tetramethrin [40], isomerisation of the putative benzene metabolite (Z, Z)-muconaldehyde to (E, E)-muconaldehyde [41], inhibition of the flavoenzymes trypanothione reductase from *Trypanosoma cruzi* [42] and thioredoxin reductase from *Plasmodium falciparum* by unsaturated Mannich bases [43], the bioactivation of alkylating agent cyclophosphamide [44], the binding of the kinase inhibitor neratinib (HKI-272) to human serum albumin [45], the inhibition of caspases [46,



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Scheme (3). Metabolic formation of thiol reactive Michael acceptor from furazolidone

47], inhibition of JNK-stimulating phosphatase-1 (JSP-1) by rhodanine derivatives [48], the inhibition of phosphofructokinase by the natural product sarcophine [49] and the *in situ* activation of the p53 activator prima-1 [50].

Many endogenous anti-inflammatory compounds and compounds involved in the resolution of tissue inflammation exert their activities by being electrophilic and possessing the capacity to covalently modify their target proteins albeit in a reversible fashion. Included in this group are Michael acceptors such as the cyclopentenone prostaglandins. Indeed, one of the key chemical structural determinants for the induction of phase 2 enzymes and anti-carcinogenic properties by small organic molecules is an electron deficient double bond, i.e. the occurrence of a Michael acceptor moiety and that the potency of these phase 2 inducers parallels their reactivity in Michael addition reactions as revealed by a structure activity relationship study using electrophilic compounds and their ability to induce quinone reductase [51]. Transduction of redox signaling related to inflammation can be regulated by electrophile-protein reactions and these most often occur with reactive protein thiols [52]. Thus thiols can be viewed as key reactive functional groups in proteins that can be covalently modified to alter protein structure and function as a response to external stimuli or changes in redox status.

The propensity for Michael acceptors to react with thiols is therefore particularly relevant to manipulating/modulating biological red/ox processes and many thiol reactive compounds, including Michael acceptors, display cytotoxic activities [53]. Michael acceptors are known to modify a variety of pathways and targets including NF-κB [54], IKK [55], Keap1 [56], peroxisome proliferators-activated in receptor gamma (PPARgamma) [57] and TRPA1 [58].

Electrophilic compounds that modulate redox signaling pathways in cells and tissue can either be xenobiotically-derived or endogenously generated through oxidative processes. Especially notable and relevant in the latter class is the formation and occurrence of electrophilic nitro-alkenes oxidatively derived from fatty acids (e.g. oleic acid and linoleic acid) that can react with cellular thiols, GSH and protein cysteine residues, through Michael additions [59]. This has been speculated to be a method of post-translational protein function through the formation of covalent bonds between protein thiols and nitro-fatty acids. GSH nitro-fatty acid adducts have been detected in healthy human red blood cells with electrospray ionization (ESI)-ion trap mass spectrometry as well as full protein nitro-fatty acid conjugates. Importantly these addition reactions with thiols are also reversible which can be viewed as a prerequisite for posttranslational modifications as these must be temporally and spatially controlled [60].

One of the most studied thiol targets in redox signaling pathways is the Keap1 protein that contains multiple reactive thiols. Induction of the Keap1/Nrf2/ARE pathway is viewed as a promising target for chemoprotection against cancer and chronic degenerative diseases [61]. Central to this approach is the targeting of key thiol residues of the sensor protein Keap1. Thiol modification of Keap leads to activation of the Nrf2 transcription factor thus activating the transcription of phase II enzymes through activation of electrophile responsive elements, EpRE. Several transcription factors containing susceptible cysteine thiols are known to undergo covalent modifications to alter their transcriptional activities [62]. These include STAT3 [63], NF-κB [64, 65] and p53 [66].

3. BIOLOGICALLY ACTIVE COMPOUNDS THAT REACT THROUGH REVERSIBLE MICHAEL ADDITIONS

There are several classes of synthetic and naturally occurring biologically active compounds where a reversible Michael addition reaction has been able to explain the pharmacological activities and cellular behavior and in some cases it has been utilized to discover new promising compounds in the form of prodrugs. Though my no means exhaustive, these examples should highlight some of the key discoveries and characteristics of biologically active reversible Michael acceptors.

3.1. Sesquiterpene Lactones

Sesquiterpene lactones are probably the most thoroughly studied naturally occurring Michael acceptors [67]. They exhibit a wide range of biological activities including anti-inflammatory and anti-cancer properties. They have been studied in various disease models but selectivity issues and safety concerns have hampered their development as drugs although they are present as constituents in many herbal remedies and traditional medicines. Their biological activities are primarily governed by the reactivity and existence of α,β-unsaturated lactone functionalities that can react with biological thiols, e.g. cysteine residues in proteins. Amongst the know targets are reactive cysteines in the NF-κB transcription pathway [8].

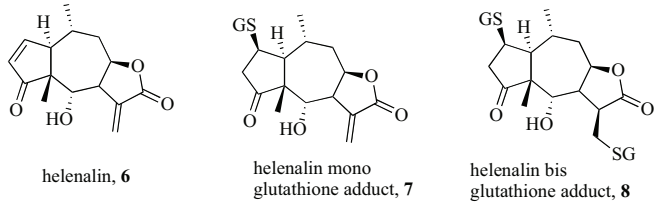
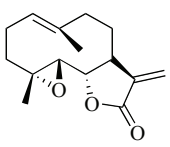


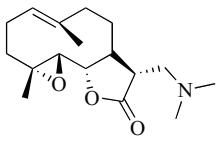
Fig. (5). The sesquiterpene lactone helenalin and its glutathione adducts.

The Michael addition of amine and thiol nucleophiles to various sesquiterpene lactones has been demonstrated to be reversible. This can in part explain the relatively low toxicity of these natural products despite their high reactivity towards biological nucleophiles. In a study aimed at understanding why the thiol reactive sesquiterpene helenalin **6** could reach its intracellular targets without being deactivated by high concentrations of intracellular glutathione, the reaction of helenalin with glutathione was monitored in a physiological buffer. Helenalin contains two reactive Michael acceptor moieties, a cyclopentenone and an α-exomethylene-γ-lactone, with the cyclopentone being significantly more reactive towards thiol nucleophiles [68]. However, at physiological pH, the Michael addition to the unsaturated cyclopentenone was readily reversible thus always allowing a small fraction

of free active compound to be available. This would explain why sesquiterpenes can display biological activities through binding to specific target proteins even in the presence high levels of glutathione. The bis-glutathion-helenalin adduct **8** displays similar inhibitory effect on the NF-κB signalling pathway as free helenalin despite lacking accessible reactive electrophilic double bonds. This can be rationalized by the fact that the addition of glutathione is reversible and at physiological pH an amount of free helanalin will always be available to react with its target molecules.



parthenolide, **9**



dimethylaminoparthenolide (DMAPT/LC-1), **10**

Fig. (6). Sesquiterpene lactone parthenolide and its dimethylamine adduct.

The existence of a reversible Michael addition has also been utilized in preparing soluble prodrugs of the sesquiterpene lactone parthenolide **9**. Parthenolide has demonstrated remarkable anti-cancer effects in several *in vitro* studies including inducing apoptosis in AML cells in a model of leukemia. However, parthenolide suffers from poor aqueous solubility and poor bioavailability. This was overcome by reacting dimethyl amine with the conjugated double exo-methylene bond in a Michael addition [69]. The dimethylamine adduct of parthenolide **10** (DMAPT, LC-1) retained its *in vitro* activity and the fumarate salt of this adduct had a thousand fold increase in solubility compared to parthenolide. Free parthenolide was not discovered in the cell lysates [46]. But in a prior study looking at the anti-hepatit C virus effects of amine adducts of parthenolide, it was found that these adducts retained the activity of the parent compound [70]. When cell-lysates were collected and analyzed by LC/MS/MS no amine adducts were detected, only parthenolide (**9**), indicating complete reversibility of the amine addition (aza-Michael addition) and replacement with other biological nucleophiles. It should be noted that a corresponding 2-mercaptoethanol adduct of parthenolide was less active. In a recent study the reversibility of amino-parthenolide conjugates was studied using ^{19}F-NMR [71]. Fluorine containing amines were reacted with parthenolide and the stability of these adducts, measured as the changes in the ^{19}F-NMR, was determined. It was found that the reversal of the Michael reaction and the release of parthenolide and amine were increased in the presence of glutathione. The reason for this was not determined but it could be due to shifting the equilibrium towards a more stable glutathione adduct as the analytical method only directly measured the release of free amine. Noteworthy is that heating at 80°C was required.

Similar effects of amine adducts of the sequiterpene lactones alantolactone **11** and isolantolactone against several cancer cell lines have been demonstrated, where the adducts show similar potencies as the parent compounds [72].

In a similar manner the reversibility of Michael acceptor adducts was previously suggested as the true source of anticancer bioactivity of ambrosin-amine covalent adducts [73]. In this study the scope of prodrugs that undergo reversible Michael additions was increased to also include sulfonates (formed through the Michael addition with bisulfonate) and sulfinate adducts (through the Michael addition of dithionite). It should be noted that the adducts were in all cases stable enough to be isolated and stored.

Other naturally occurring compounds that can be viewed as prodrugs of Michael reactive sesquiterpene lactones have since then been discovered.

An important observation in the study of different sesquiterpene Michael adducts, is that the reversibility of amine and thiol Michael adducts of sesquiterpene lactones differs and their stability seems to depend on the structure of the Michael acceptor. Thiol nucleophiles, e.g. glutathione, bound to cyclopentone moieties of sesquiterpenes seem to more readily undergo retro-Michael additions than when bound to the unsaturated exomethylene bond of α-exomethylene-γ-lactone groups [74]. This is in contrast to the reversibility of the amine adducts of α-exomethylene-γ-lactones, as witnessed by their propensity to undergo retro-Michael additions in physiological media and thus can serve as water-soluble prodrugs. Thiol conjugates of α-exomethylene-γ-lactones seem to be less active.

In addition to the spontaneous reversible Michael addition there is also the possibility of enzyme catalyzed retro-Michael additions with glutathione transferase enzymes or through a stepwise procedure with oxidation of sulphur to the corresponding sulfoxide followed by spontaneous or base-catalyzed syn elimination. It has been postulated that cellular oxidative processes or the presence of hydrogen peroxide can further increase the liberation of free and active α,β-unsaturated lactone from the corresponding Michael acceptor-thiol adducts. Here the mechanism is initial oxidation of the sulphur atom to the corresponding sulfoxide followed by retro-elimination of the thiosulphenic moiety. This has been proposed as a strategy to design pro-drugs of unsaturated sesquiterpene lactone that are selectively unmasked in an oxidative cellular environment [75].

The syn elimination of sulfoxides is speculated to be the source of biological activity of sulphide derivatives of the α,β-unsaturated lactone containing antibiotic Brefeldin A (**15**) [76] The sulfoxide (**17**) analogs display higher *in vitro* activities than the corresponding sulfides (**16**) as they are thought to be reverted to the biologically active component brefeldin A. The direct elimination of the sulfides through a retro Michael addition was not observed.

3.2. Electrophilic Cyclopentenone Prostaglandins

Prostaglandins containing an electrophilic cyclopentenone ring system, such as Δ^{12}-PGJ$_2$ (**18**) and Δ^{7}-PGA$_1$ (**19**), display unique anticancer [77] and anti-inflammatory effects [78]. Structure activity relationships studies of these compounds clearly show the need for a cross-conjugated dienone system to achieve potent anti-proliferative effects

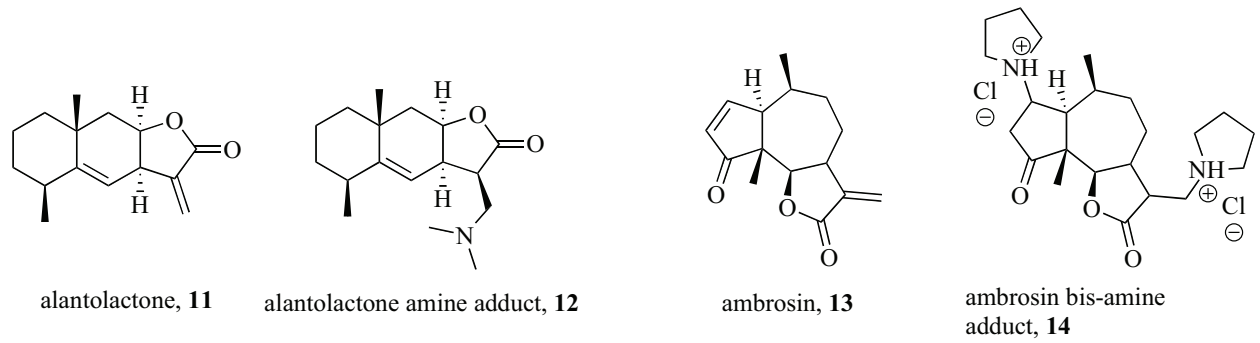
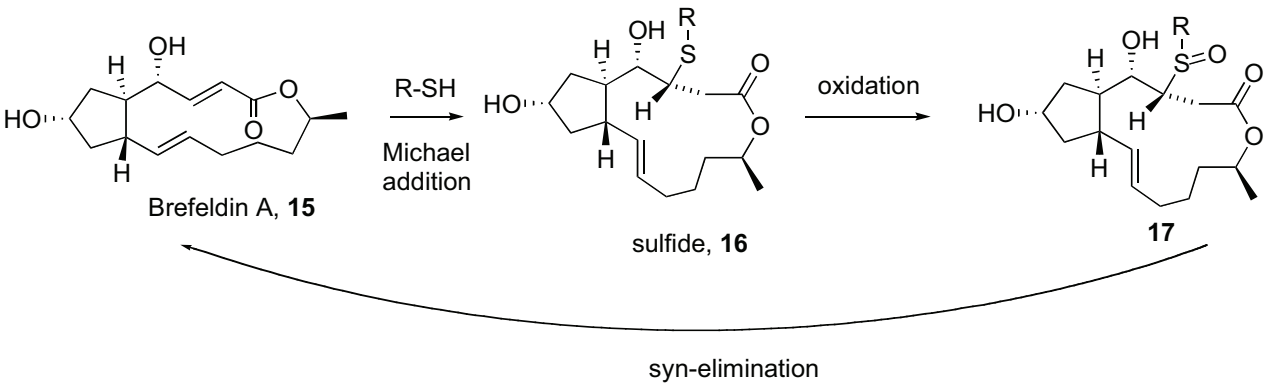


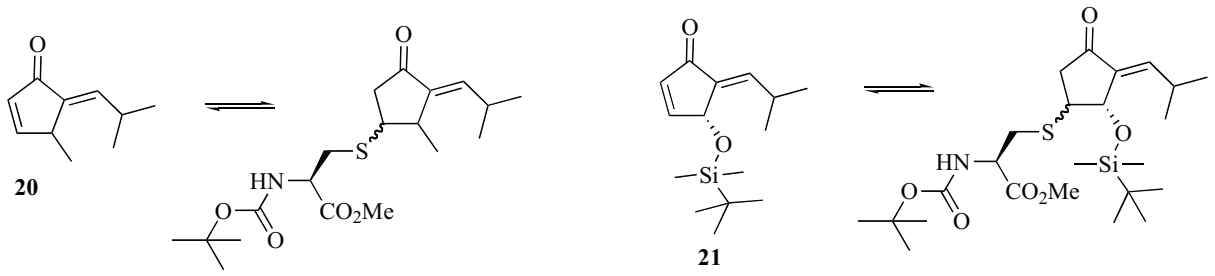
Fig. (7). Sesquiterpene lactones and their biologically active amine adducts.



Scheme (4). Formation and reactivity of Brefeldin A prodrug.

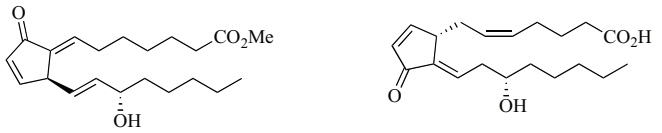
[56]. The biologically activity of these compounds is clearly related to the existence of the α,β-unsaturated cyclopentenone functionality that can act as a Michael acceptor towards nucleophiles. These prostaglandins react readily with thiol nucleophiles, including, glutathione, in physiologically relevant conditions (CD_3OD-deuterio phosphate buffer, pH 7.4) [79]. Importantly this reaction is reversible, establishing an equilibrium between the adduct and the corresponding free cyclopentenone prostaglandin and free thiol. The thiol-prostaglandin adducts were stable enough to be isolated but underwent retro-Michael addition under the conditions described above. The existence of the cross-conjugated system increased the forward reaction rate compared to the corresponding simple mono-conjugated cylopentenone but these compounds also undergo the reverse reaction more readily, i.e. the thiol-adduct is more labile, and thus exists in its free unbound state to a larger extent under thermodynamic conditions. The equilibrium is also highly dependent on pH; at pH 7.4 the prostaglandin-glutathione adduct dissociates readily, reaching a 1:1 equilibrium after 15 min but at pH 6.0 the reverse reaction was very slow. The propensity of cyclopentenone prostaglandins to undergo Michael additions can explain their biological activities but the reversibility of the addition reaction can also explain their cellular behaviour. It is believed that free unbound cyclopentenone prostaglandin is transported into the cells where it will react with intracellular glutathione. But because of the reversibility of the thiol Michael addition, there will always be free cyclopentenone prostaglandin that can enter the nucleus and bind to its target proteins and exert its biological effect. It is then believed that the protein- cyclopentenone prostaglandin adduct will be more stable, i.e. less susceptible to undergo retro Michael addition, due to decreased molecular motion in this covalent super-molecular complex with the target protein. Other stabilizing interactions with the target can also play a part in reducing the reversibility, e.g. changes in local pH. However, electrophiles that enter the cell and react irreversibly with intracellular glutathione (present in concentrations of 1-10 mM) will be actively transported out of the cell as their glutathione adducts by efflux pumps such as MRP/GS-X. Thus by shuttling between bound and free form, an electrophile can be selective and end up binding to its target protein providing that stabilizing interactions are present in the protein-inhibitor covalent complex allowing this to be the thermodynamically most stable adduct.

The reversibility of the Michael addition to cyclopentenone prostaglandins has also been studied using simplified cyclopentenone prostaglandin analogs (**20** and **21**) [80]. Competition experiments showed that glutathione could be replaced with cysteine and that thiol adducts were retained the ability of the mother compound in inhibiting NF-κB signalling indicating that they undergo retro-Michael reactions to give free and reactive cyclopentenone. Interestingly the authors speculate that the hydrophobicity of



Scheme (5). Reversible thiol addition to cyclopentenone prostaglandin analogs.

the conjugated thiol can influence the rate and intracellular location of the retro-Michael addition.



D^{12}-PGJ_2, **18** D^{7}-PGA_1, **19**

Fig. (8). Cyclopentenone prostaglandins.

3.3. Ethacrynic Acid

Ethacrynic acid (**22**), a diuretic drug containing an α,β-saturated ketone functionality, is a potent and reversible inhibitor of GST (glutathione *S*-transferase) isoenzymes. **22** has been shown to be able to undergo reversible Michael addition reactions with thiol nucleophiles. When the ethacrynic acid glutathione adduct (resulting from a Michael addition) was incubated with an excess of *N*-acetyl-L-cysteine, this amino acid was transferred to ethacrynic acid, replacing GSH [81]. This transfer was pH dependent and equilibrium was reached. This transfer also worked when the cysteine residue was present in a protein. A [^{14}C]-labelled GSH-ethacynic acid adduct transferred [^{14}C]-ethacrynic acid to the GST P1-1 enzyme which contains a reactive cysteine moiety (Cys-47) presumably through an initial retro-Michael addition releasing reactive ethacrynic acid. The activity of the covalently inhibited protein could be restored upon incubation with GSH (excess). This raises the possibility of transport of bioactive Michael acceptors as thiol-conjugates thereby masking their reactivity. Following subsequent regeneration and unmasking of the Michael acceptor functionality through a retro Michael addition the biologically active compound is revealed. This could be utilized in the design of Michael acceptor prodrugs. It should also be possible to improve the physical chemical properties through conjugation and adduct formation as the adduct, through judicious selection of nucleophile, could display higher aqueous solubility and resistance to metabolic processes.

Ethacrynic acid (**22**) has also been evaluated as a drug for the treatment of glaucoma and ocular hypotension but poor ocular bioavalibility has precluded further development. An analog of ethacrynic acid, SA9000 (**23**), demonstrated significantly improved corneal penetration and showed promising *in vivo* effects. However, this was accompanied by ocular irritation. Conjugation with cysteine to afford a prodrug of SA9000 (**24**) that could undergo retro Michael addition in the desired target compartment might alleviate this problem. Indeed, the cysteine adduct prodrug **24** did produce less irritation and it was shown that it had a more beneficial distribution giving less extraoccular exposure while retaining efficacy [82].

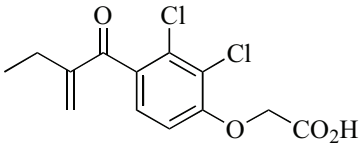


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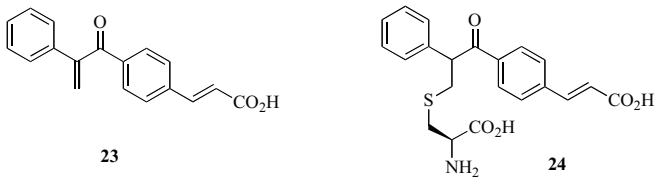
Fig. (9). Ethacrynic acid.



23 **24**

Fig. (10). Ethacrynic acid analog SA9000 and SA9000-cysteine prodrug.

A very ingenious use of the reversibility of the Michael addition between ethacrynic acid and thiols was utilized in the construction of a dynamic combinatorial library of GSH tranferase (GST) inhibitors where the pH dependency of the reverse reaction was used to select and molecularly amplify GST inhibiting adducts [83].

3.4. Bardoxolone

Perhaps the most significant discovery that highly reactive Michael acceptors can be safe and efficacious is the discovery and development of the synthetic oleanane terpenoid bardoxolone **25** (CDDO-Me, RTA402) [84]. Bardoxolone is a highly potent anti-inflammatory compound as evident by its capability of inhibiting inducible nitric oxide synthase (iNOS) at sub nanomolar concentrations in macrophages [85]. The desirable safety profile of this compound [86] has been rationalized by the reversibility of thiol adduct formation. **25** reacts readily with thiol nucleophiles (glutathione, DTT) but these adducts are not

stable and were not isolable and could only be detected using spectroscopic methods [87]. In an apparent contradiction biotinylated bardoxolone analogs have been used to identify and isolate target proteins through streptavidin purification. The identified targets include IKKβ [88], JAK2, STAT3 [89] and mTOR [90]. These covalently bound bardoxolone-protein adducts must therefore be stable enough, i.e. the reversibility of the Michael addition is low, to allow protein capture and identification. This lends further credence to that macromolecules provide additional stabilizing interactions that are not present with simple low molecular weight nucleophiles. This has been termed a "dock and lock" mechanism.

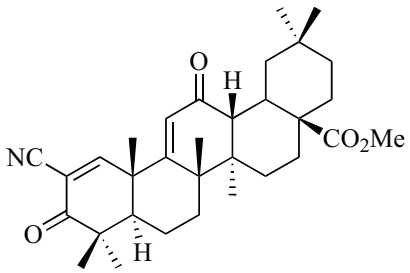


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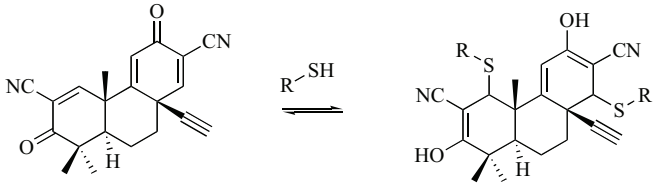
Fig. (11). Bardoxolone (CDDO-Me)



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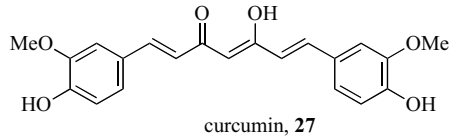
Scheme (6). Thiol reactive tricyclic bardoxolone analog

However, there are many indications that bardoxolone and simplified tricyclic cyano enone analogs (e.g. **26**) of bardoxolone bind to various cysteine residues on Keap1, yet the protein-inhibitor adducts have not been isolated presumably due to the reversibility of the Michael addition, suggesting that no significant stabilizing interactions exert their actions and that the covalent interaction is transient and reversible, at least under the conditions for protein purification. The reversibility of formation of Michael adducts with these simplified tricyclic cyano enone analogs of bardoxolone (**26**) has also been speculated to be a reason for their potent *in vivo* effects despite their high reactivity towards nucleophiles [91].

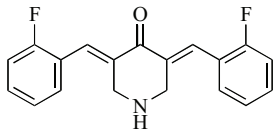
The lack of toxicity and the proven safety of bardoxolone show that highly reactive Michael acceptors can be tolerated and that the reversibility of the formation of a covalent bond could be a reason for this. Actually the reversibility of the Michael addition of bardoxolone and simplified analogs is one of the key factors of their unique biological properties, e.g. allowing pulsed activation of anti-inflammatory pathways and reversible modification of Keap1, thus circumventing the need for de novo protein synthesis, and also by enhancing the bioavailability and preventing their inactivation even in the presence of high concentrations of glutathione. Bardoxolone is now being evaluated in phase II clinical trials for the treatment of chronic kidney disease.

3.5. Curcumin

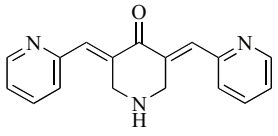
Curcumin (**27**), a constituent of the spice turmeric, has been used in traditional medicine for centuries but has gained increased attention due to it's anti-cancer, anti-inflammatory and anti-angiogenic effects. Among the targets of this phytochemical is NF-κB [92]. Despite displaying a wide range of relevant *in vitro* effects and low toxicity, curcumin's clinical development as a drug has been hampered by poor drug-like properties such as low aqueous solubility and inadequate permeability. Curcumin **27** is a bis-α,β-unsaturated ketone that can react as a Michael acceptor with thiols and this reactivity is thought to be responsible for many of curcumin's biological effects [93]. Biologically active synthetic curcumin analogs often retain the Michael acceptor functionality. Two such analogs, EF24 (**28**) and EF23 (**29**), retaining the bis α,β-unsaturation, display increased activity compared to curcumin, inhibiting NF-κB translocation at 10-fold lower concentrations. However, these analogs still display poor aqueous solubility. In an attempt to overcome this bis-glutathionyl adducts were prepared. These adducts could be isolated as stable solids with increased aqueous solubility. When tested against MDA-MB-435 breast cancer cells, the adducts displayed the same cytotoxic activity as the mother compounds. This is explained by the bis-glutathionyl adducts undergoing retro-Michael addition to release the active the compound. Thus thiol adducts of curcumin or curcumin analogs could potentially be used as prodrugs thus circumventing the limitations of curcumin.



curcumin, **27**



EF24, **28**

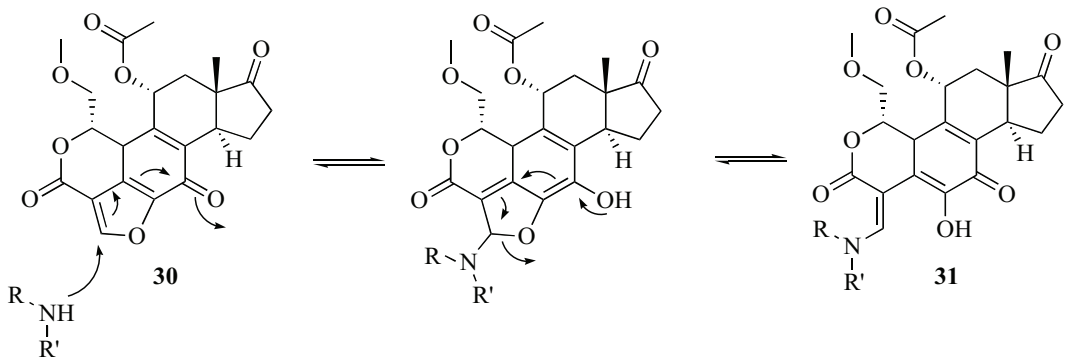


EF23, **29**

Fig. (12). Curcumin and simplified analogs EF24 and EF23.

3.6. Viridins

The viridins are a group of natural products with a range of biological activities including anti-inflammatory and anti-cancer properties [94]. Key to their biological activity is the presence of an electrophilic double bond, part of fused furan, that is doubly activated by a lactone and a vinylogous α,β-unsaturated ketone. Wortmannin (**30**), a member of the



Scheme (7). Reversible reaction of wortmannin with amines.

viridin family, is a potent inhibitor of PI3 kinase [95]. Despite being highly reactive and seemingly instable in the presence of biological nucleophiles in culture media, wortmannin still displays potent *in vivo* activity. This has been termed the wortmannin paradox [96]. This can be explained by the reversible nature of the conjugated addition of nucleophiles, e.g. amino acids, to wortmannin (Scheme **7**) [97]. Wortmannin amine adducts (**31**) are stable and water-soluble. Thus wortmannin can be released in its active form in or near the active site of its target.

4. DISCOVERING AND DESIGNING REVERSIBLE MICHAEL ACCEPTORS

An NMR method to specifically and quickly assess the reversible nature of thiols additions to Michael acceptors has been developed [98]. However, the authors define a stringent view of what constitutes a reversible and an irreversible Michael reaction by defining a reversible Michael reaction as the case where the adduct cannot be isolated from the reaction medium. This is in contrast to the possibility of isolating thiol adducts of for example cyclopentenone prostaglandins that are evidently capable of undergoing reversible Michael addition reactions (vide supra). The described NMR method utilizes the solvent dependence of the Michael addition where the addition is promoted by a polar aprotic solvent, e.g. DMSO. By switching from DMSO to chloroform reversible adducts will undergo a retro-Michael reaction while adducts of non-reversible Michael-additions will be stable. In this way the reversible Michael additions can be detected. The assay was able to confirm the lack of reversibility of sesquiterpene lactone thiol adducts where the thiol has reacted with the exomethylene bond. This method could become a useful assay for finding chemical scaffolds that act as reversible Michael acceptors but, as the authors state, more studies need to be conducted to understand the mechanism behind the reversibility and what the factors that influence the reversibility are.

Another predictive NMR method was developed to discover potential Michael acceptors among a class of pyrazolinones as KDR inhibitors [99]. The ^{13}C NMR shifts of the β-carbon of the α,β-unsaturated carbonyl moiety correlated well with the Michael acceptor activity of the compounds. The compounds with higher ^{13}C NMR shifts β-carbon shifts (137-139 ppm) displayed a greater reduction in KDR inhibition potency when DTT was added to the assay system indicating that these reacted as Michael acceptors forming inactive adducts and thus losing activity. Higher shifts indicated an increased likelihood of Michael addition. The extent of the reversibility of the addition was not correlated with the NMR shifts. But the electrophilicity of the conjugated double bond as measured by ^{13}C NMR shifts cannot be used solely as a predictor of activity as witnessed previously among a group of electrophilic bis(benzylidene) acetones [100].

5. CONCLUSION

We believe it is very important to understand the nature and molecular mechanism of action of biologically active covalent inhibitors; not all covalent inhibitors are the same. The covalent inhibition can be reversible or irreversible and this will have a profound effect on the selectivity, toxicology and efficacy of the inhibitor. The reversible nature of the Michael addition is governed by many factors including the structure (electronics and sterics) of the Michael acceptor and the stability of the adduct as well as the surrounding conditions (concentration of competing nucleophiles, pH, solvent, target binding etc). These areas require more study to enable the rational development of safe and efficacious reversible covalent inhibitors with high reactivity. Factors that need to be studied are the interactions that stabilize reversible Michael acceptor adducts in their target proteins. Are these due to local conditions in the target-inhibitor adduct; changes in pH or solvent polarity, or are there specific stabilizing interactions (lipophilic, hydrogen bonding etc) when the inhibitor is bound to the target or is it due to reduced molecular mobility? Is there a dependency on the localization of the target and the target-inhibitor adduct? It has been speculated that covalent inhibition of intracellular targets would provide a reduced risk of iodiosyncratic adverse events caused by an immunogenic response compared to covalent inhibition of extracellular targets as the intracellular binding event would not be presentable as an antigen and thereby less effective in stimulating an immune response. Therefore it is possible that the equilibrating thermodynamic nature of the reversible Michael addition would allow the inhibitor to be concentrated in the intracellular milieu.

Another possibility is that there are cases where no stable protein adducts are formed with the covalent inhibitor, but instead the reversible reactive compounds act as modulators

just causing perturbation of signaling pathways in a manner similar to covalent posttranslational protein modifications. An example of this could be the reaction with key thiol groups in signaling proteins thus offsetting the thiol redox balance of the signaling pathway. With an increased interest in compounds that do affect thiol redox signaling and ROS production in cancer cells either through known thiol reactivity (CDDO) or suspected thiol addition properties (piperlongumine [101]) it is warranted with further studies of the nature of the reversible reaction of thiols with electrophilic compounds containing Michael acceptor functionality.

Electrophilic Michael acceptors can react reversibly with abundant biological nucleophiles, e.g. cysteine and glutathione, and this reactivity does not in itself appear to constitute a direct cause of toxicity. The rate and extent of reversibility can be and has been studied and some of the factors affecting the reverse reaction (i.e. the pKa of the adduct) are known. However, the non-covalent interactions of covalently bound inhibitors within biological macromolecules, proteins, need to be further studied to understand what factors influence the extent of the reversibility, i.e. is the formed adduct stabilized through other interactions. A crystal structure of a known reversible Michael acceptor inhibitor bound to its target protein would be very valuable.

In light of what is known about the role of posttranslational modifications of signaling proteins to modulate pathways related inflammation, reactive, but reversible, Michael acceptors, that can act as electrophiles mimicking the action of endogenous electrophilic compounds that can resolve inflammation and induce the expression of anti-carcinogenic proteins, could be an important source of drug-like compounds. These could achieve high potency and efficacy through the formation of covalent bonds and, depending on the covalently bound compound-target complex (i.e. do additional stabilizing factors exist), they could either be long acting or act in a rapid and transient fashion.

The occurrence of specific accessible thiols in key signaling proteins, especially in redox signaling pathways related to inflammation and carcinogenesis that can be covalently modified endogenously for modulating cellular signals should make these proteins and their reactive thiols important targets for pharmacological interference with covalently acting drugs.

During the preparation of this manuscript Jack Taunton and coworkers of University of California, published their work on developing reversible covalent inhibitors of the p90 ribosomal protein S6 kinase RSK2 through targeting a non-catalytic cysteine with a chemically tuned electrophilic moiety [102]. By increasing the reactivity of an acrylamide moiety through the addition of a second electron-withdrawing group (a nitrile) a more thiol reactive compound was obtained. But in line with what has been discussed in this review, the increased reactivity was also followed by increased reversibility of the thiol addition reaction. A cocrystal structure of the covalently bound inhibitor showed how secondary interactions stabilized the complex and disruption of these interactions led to reversal of the covalent bond formation. This work very elegantly demonstrates how reversible covalent Michael acceptors can be designed as inhibitors and how they might have the potential to overcome some of the limitations and risks of irreversible inhibitors while still providing long lasting biological effects.

CONFLICT OF INTEREST

The author declares no conflict of interest or competing financial interest.

ACKNOWLEDGEMENTS

The author is grateful to Professor Anders Bjartell and the Department of Urology and Center for Molecular Pathology at Skåne University Hospital and Lund University for financial support and funding and to Lisa Johansson for comments on the manuscript.

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Received: January 16, 2012 Revised: May 09, 2012 Accepted: May 15, 2012