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Covalent inhibition has a rich history in drug discovery and continues to be a highly successful strategy for addressing diverse targets and disease areas.

Covalent inhibitors in drug discovery: from accidental discoveries to avoided liabilities and designed therapies

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Drugs that covalently bond to their biological targets have a long history in drug discovery. A look at drug approvals in recent years suggests that covalent drugs will continue to make impacts on human health for years to come. Although fraught with concerns about toxicity, the high potencies and prolonged effects achievable with covalent drugs may result in less-frequent drug dosing and in wide therapeutic margins for patients. Covalent inhibition can also dissociate drug pharmacodynamics (PD) from pharmacokinetics (PK), which can result in desired drug efficacy for inhibitors that have short systemic exposure. Evidence suggests that there is a reduced risk for the development of resistance against covalent drugs, which is a major challenge in areas such as oncology and infectious disease.

Introduction

Most small-molecule drugs are designed to interact with their biological targets under equilibrium binding conditions, where the desired drug–protein interaction is a rapid and reversible process. The ratio of a drug–protein complex to unbound drug and free protein is dependent on the intrinsic affinity between the two partners. Given that the interaction of a drug with its target is, in aggregate, the phenomenon that leads to a therapeutic response, a common focus of modern drug discovery programs is to maximize the strength of these noncovalent molecular interactions. However, as the enterprise of drug discovery advances, a nonconventional strategy termed 'covalent inhibition' has permeated the consciousness of an increasing number of drug-hunting teams. In covalent inhibition, a small molecule is designed not only to bind to a protein through traditional reversible interactions, but also to undergo a bond-forming event that produces a durable drug–protein linkage. The resulting covalent bond can be sufficiently long lived that it is irreversible within the half-life of the target protein, resulting in a drug–protein complex that is not subject to classical equilibrium kinetics (Fig. 1). In contrast to conventional drugs, irreversible inhibitors can theoretically achieve complete neutralization of biomolecular targets given enough time. As a result, covalent inhibitors are not easily ranked using traditional IC_{50} measurements that can be time dependent, and instead require a consideration of the rate of inactivation of a target (k_{inact}, Fig. 1). Thus, the k_{inact}:K_i ratio is normally preferred over IC_{50} values to rank the potency of covalent inhibitors against a target [1,2].

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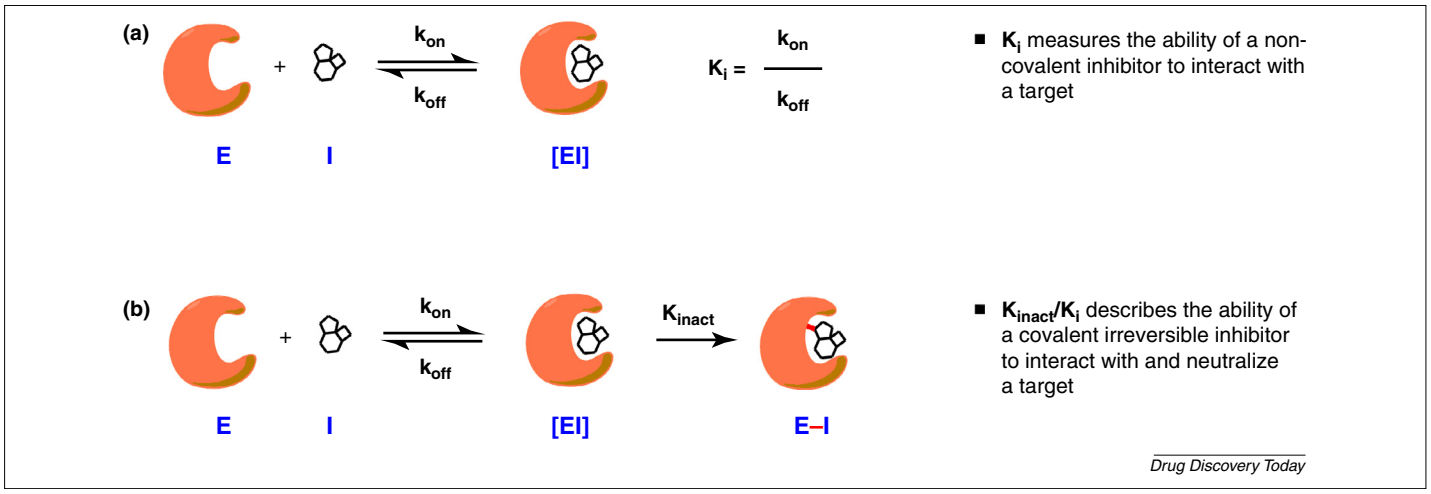

FIGURE 1

Comparison of an inhibitor (I) interacting with an enzyme (E) under **(a)** traditional non-covalent and **(b)** covalent binding manifolds.

In 2005, Robertson published a widely cited analysis on the mechanistic basis of enzyme-targeted drugs, noting that 'drug discovery programs never set out to make irreversible inhibitors' [3]. Despite this conventional wisdom, Robertson also found that 26% of the 71 enzymes analyzed were covalently inhibited by approved drugs. Indeed, observations similar to this abound in the literature, illustrating the astonishing contrast between the potential of covalent inhibition in drug discovery and the simultaneous bias against such agents during development [4]. Today, the idea that covalent drugs can contribute to improving human health is becoming more widely appreciated, as reflected in drug approvals and in recent literature citations [5–10]. The number of publications that reference covalent inhibitors has recently increased, seemingly in an exponential manner, even when normalized against all drug discovery manuscripts to account for an increase in new journals over time (Fig. 2). With this context in mind, I review here the development of covalent inhibitors to treat human disease, with a focus on historical framework, advantages, obstacles, clinical examples, and physicochemical features.

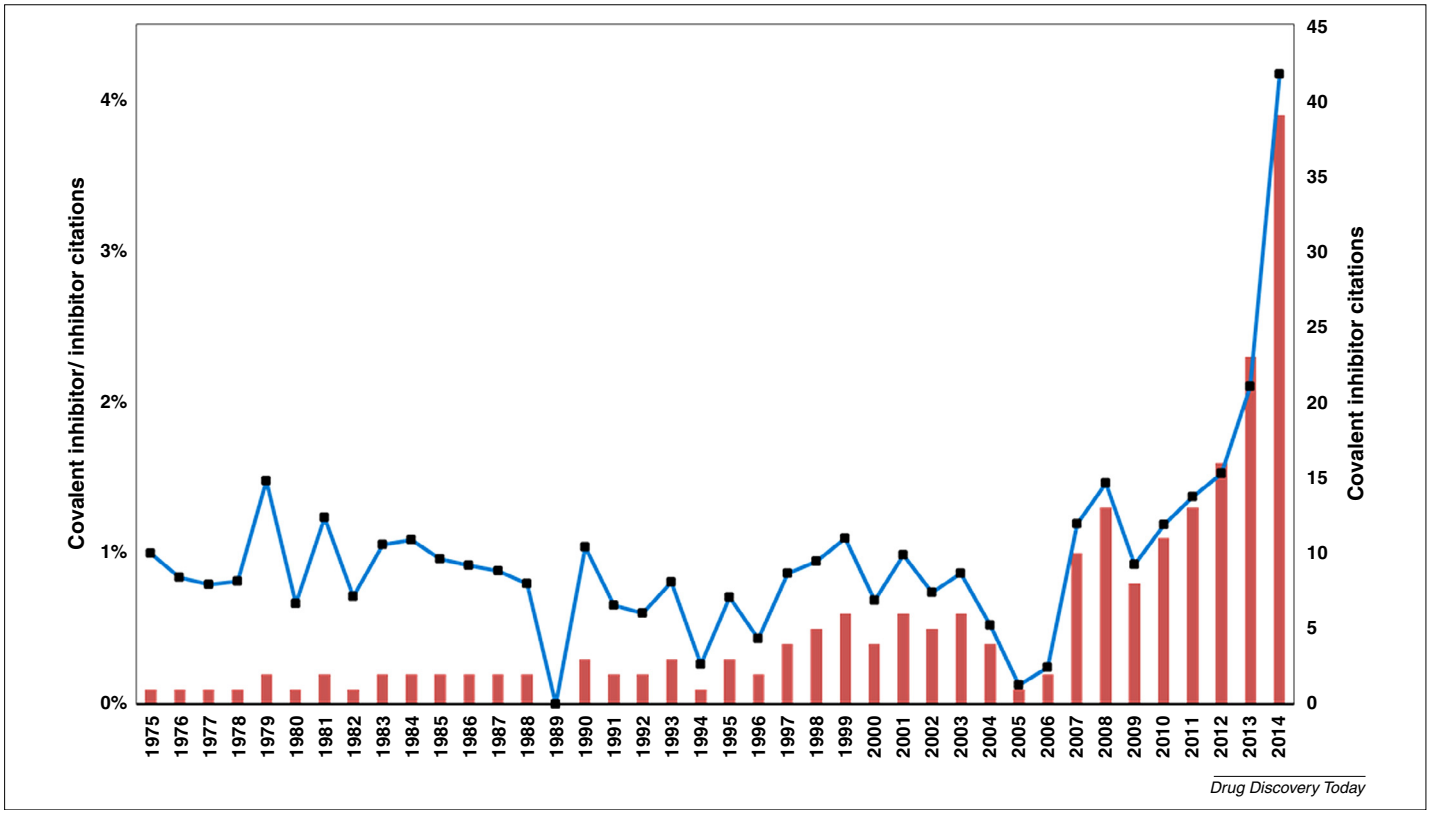

FIGURE 2

Chart of Scifinder citations containing the phrase 'covalent inhibitor' from 1975 to 2014 (red bars). Normalization against all inhibitor citations is overlaid (blue line).

Historical framework

The use of small-molecule drugs to covalently inhibit biological targets dates back to the beginnings of drug discovery, when Bayer during the late 1800s began investigating and manufacturing aspirin (acetylsalicylic acid) to treat pain and inflammation. Remarkably, it was not known until the 1970s that aspirin acts by the covalent and irreversible inhibition of cyclooxygenase (COX)-1 and -2, enzymes responsible for the biosynthesis of prostaglandins [11]. Beyond aspirin, the serendipitous development of other covalent inhibitors has a storied past, as highlighted by famous drugs that include antibiotics such as the penicillins [12], the cephalosporins [12], and fosfomycin [12]; and agents whose metabolites are covalent inhibitors such as omeprazole and lansoprazole (proton pump inhibitors) [13], and clopidogrel (antiplatelet) (Fig. 3) [14]. Today, there are at least 42 approved covalent drugs used to treat ailments ranging from obesity to aggressive malignancies [4]. Although most covalent drugs target enzymes, receptors such as G protein-coupled receptors (GPCRs) have also been drugged covalently (e.g., using clopidogrel). Interestingly, over the course of evolutionary history, selection pressure has resulted in a range of natural products that act on protein targets covalently [15]. Valuable insights from the chemistry of these natural products have led to the design of derivatives that are currently in clinical trials or that are already approved. Although many covalent natural products are innately cytotoxic (a feature significant to anticancer and anti-infective research), medicinal chemistry efforts have also delivered natural product-inspired therapies that are not overtly cytotoxic, such as orlistat (obesity), beloranib (obesity), and rivastigmine (dementia). In addition, the potential of covalent drugs has recently inspired the creation of numerous biotechnology companies that focus exclusively on the development of this drug class [16,17]. Given the rich history of covalent drugs, practitioners of drug discovery might be well served to weigh the potential advantages and disadvantages of covalent inhibition for different disease areas and associated targets.

Covalent inhibitors as avoided liabilities

A widespread view in drug discovery is that electrophiles should be excluded from drug candidates for reasons primarily relating to safety [18]. The tendency to avoid electrophilic functionalities in medicine tracks to some extent with the research of James and Elizabeth Miller during the mid-20th century on reactive metabolite theory [7,19]. Over their decades-long studies on the toxic effects of xenobiotics, the Millers found strong associations between a variety of simple chemicals and carcinogenesis (e.g., *N*, *N*-dimethyl-4-aminoazobenzene and *N*-acetyl-2-aminofluorene). The Millers postulated that certain chemically inert chemicals are converted by the metabolic machinery of the body to electrophilic metabolites that subsequently react with proteins, lipids, DNA, and other biomolecules to cause cellular damage (Fig. 4).

Other observations during the second half of the 20th century also conspired against the use of electrophiles in drugs. During the 1970s, the acute toxicity resulting from large doses (i.e., several grams) of acetaminophen was traced to *N*-acetyl-*p*-benzoquinone imine (NAPBQI), a primary drug metabolite [20]. NAPBQI is a

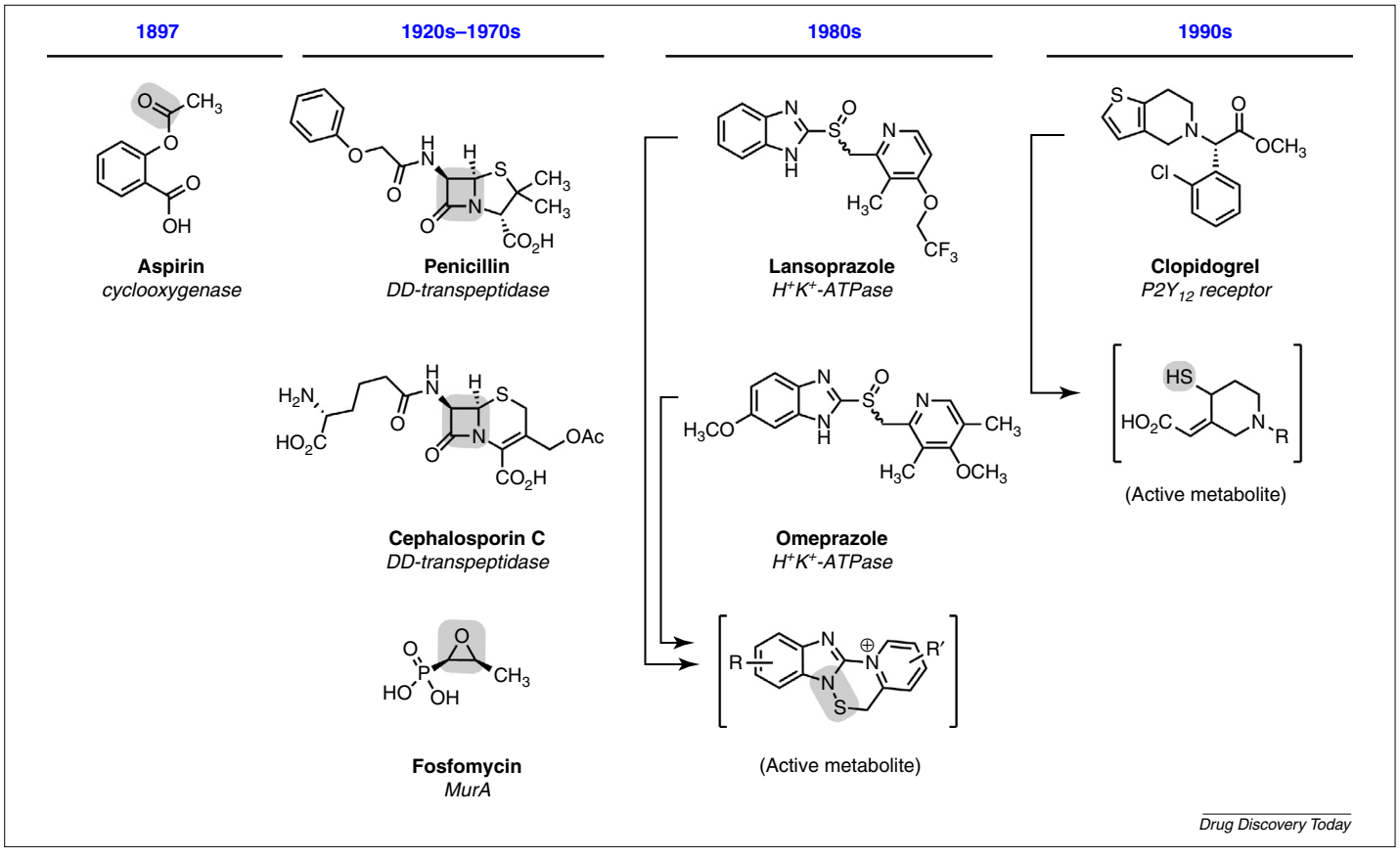

FIGURE 3

Historical examples of approved covalent drugs. Electrophiles are highlighted (sulfhydryl, SH, is a pre-electrophile).

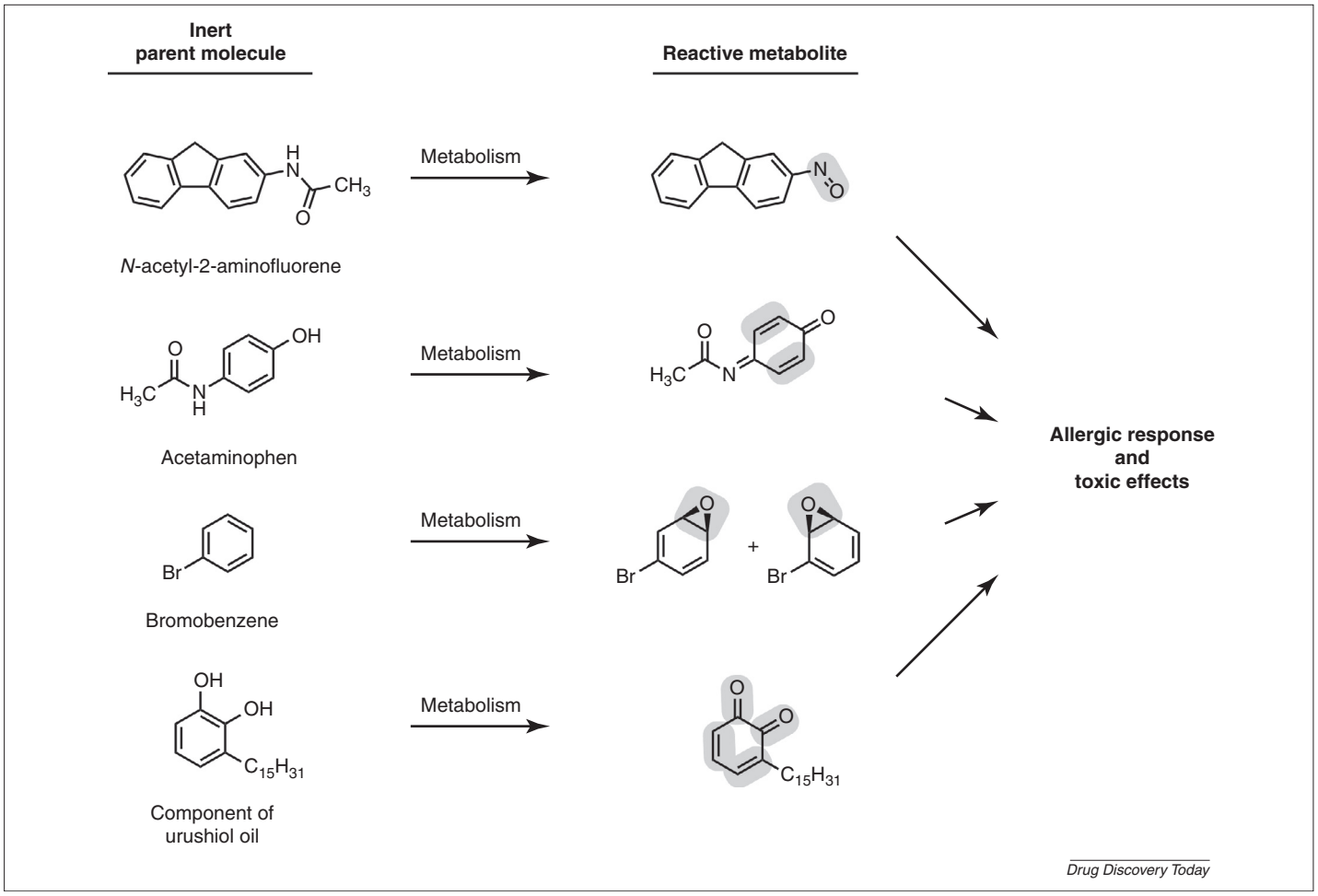

FIGURE 4

Examples of xenobiotic reactive metabolites that cause adverse effects such as allergies, tissue destruction, or carcinogenesis. Electrophiles are highlighted.

promiscuous electrophile that readily reacts with circulating nucleophiles such as glutathione and various hepatic proteins. The acute toxicity of NAPBQI is so powerful that suicide by acetaminophen overdose is not uncommon worldwide [21]. Bromobenzene is another widely characterized reagent (although not a medicine) whose primary metabolite is a potent alkylator of macromolecules. The toxic metabolites 2,3- and 3,4-bromobenzene epoxide are formed by the processing of bromobenzene by mixed function oxidases in the liver and are known to cause significant tissue damage [22]. Although the liver is commonly the target organ of reactive metabolites, other organs, such as the skin, are also vulnerable. Urushiol, an oily substance produced by certain plants including poison ivy, is readily oxidized *in vivo* to generate electrophilic *ortho*-quinones that react with nucleophilic amines and thiols on membrane proteins [23]. The haptenization of host proteins by urushiol quinone causes the commonly observed dermatitis seen at the site of contact, resulting from activation of the immune system.

Examples such as acetaminophen, bromobenzene, and urushiol have raised concerns around the use of electrophilic functional groups, such as Michael acceptors and epoxides, in drugs for sound reasons. However, although these reagents can be indiscriminate alkylators of biomolecules, less-reactive electrophiles, such as acrylamide- and nitrile-containing drugs, are generally safe and are used clinically with much success. Therefore, it is suggested that careful evaluation of electrophilic functionality is essential before ruling out chemical matter during a discovery campaign. For example, in comparing two chlorinated classes of molecule, the sweetening agent sucralose and the nitrogen mustard drug class, one finds notable structural similarities (Fig. 5). However, the

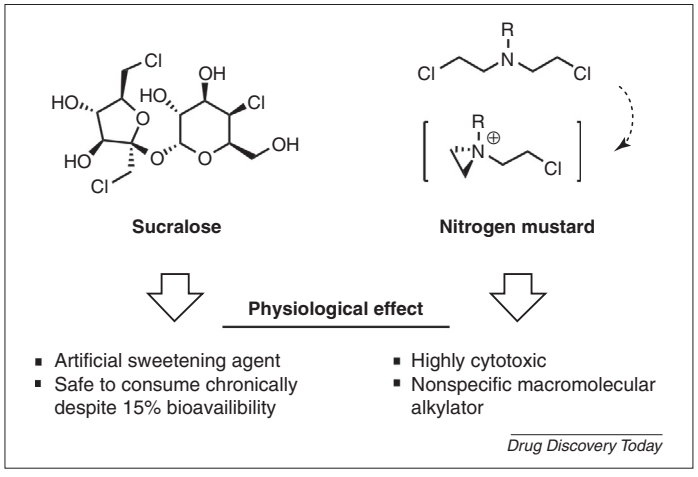

FIGURE 5

Two agents containing inert versus reactive alkyl chlorides.

different local environments of the alkyl chlorides found in these agents result in unique biological effects. Whereas sucralose is safe to consume chronically as a food-sweetening agent [24], nitrogen mustards are reserved only for the most serious cancer indications because their hyper-reactive chlorides and associated aziridiniums result in nonspecific alkylation of macromolecules, such as DNA and plasma proteins [25].

Drugs with moderate or poor electrophiles also carry toxicity risks, even when preclinical models fail to show evidence of wholesale macromolecular alkylation. Amoxicillin, a β-lactam antibiotic, contains a mild electrophile that is considered relatively safe but which causes dangerous adverse effects in some patients. Toxicity such as that derived from amoxicillin is idiosyncratic in nature because it is not predicted preclinically and is not derived from the primary pharmacology of the drug [26]. Idiosyncratic toxicity is often explained by the reaction of a drug or its metabolite with proteins to create foreign epitopes that activate the immune system of the host, even at low levels (Box 1).

Of course, idiosyncratic toxicity is a concern for all drug development programs, irrespective of whether a drug is covalent or noncovalent by design. Historical examples of agents whose pharmacology is noncovalent but which still cause idiosyncratic toxicity include halothane (anaesthetic), sulfamethoxazole (antibiotic), carbamazepine (antiepileptic), and felbamate (antiepileptic) [27]. By contrast, many approved covalent inhibitors have been used safely for decades with no observed idiosyncratic toxicity. An alternative, widely cited factor that is strongly associated with risk of toxicity is the daily dose of a drug [26,28,29]. The risk of idiosyncratic toxicity appears to be proportional to this daily dose; at doses less than 10 mg per day idiosyncratic toxicity is rare, regardless of the mechanism of a drug [26,30].

BOX 1

Summary of pros and cons for covalent inhibitors

Pros

- High biochemical efficiency may translate to lower doses and reduced off-target effects
- Nonequilibrium binding might help to overcome competing endogenous substrate concentrations that bind to the same target site
- Covalent binding might mitigate the development of drug resistance resulting from mutation of a binding site.
- Uncoupled PK/PD and prolonged duration of action can result in less-frequent drug dosing
- Can potentially address targets with shallow, undruggable binding sites

Cons

- Potential risk of idiosyncratic toxicity and/or immune-mediated drug hypersensitivity
- Hyper-reactive warheads might lead to other drug-induced toxicity (e.g., hepatotoxicity, mutagenicity, or carcinogenicity)
- Not suitable for mechanisms requiring short residence time, transient or partial inhibition
- Little advantage for biological targets that are rapidly turned over by protein synthesis

Advantages of covalent drugs

Unacceptable toxicity and insufficient efficacy are frequently cited reasons for the attrition of drug candidates during large-scale clinical trials [31]. While pharmaceutical teams continue to hedge against attrition by careful target and patient selection [32], the main defense of medicinal chemists against attrition is to synthesize drug-like molecules that are maximally potent and selective for their desired targets. Small molecules that engage their targets covalently have been found to exhibit uniquely high levels of biochemical efficiency; within approved drugs, this feature correlates with high efficacy and favorable therapeutic margins [33].

It is not unusual for conventional noncovalent drugs to suffer from decreased potency as endogenous substrates build up during therapy and compete for target binding [33]. Swinney estimates that the biochemical mechanisms of 80% of approved drugs result in competition with endogenous ligands that interact with the same protein-binding site [34]. By contrast, because covalent inhibitors operate under nonequilibrium binding kinetics, an advantage of their use is the mitigation of any potential competition with endogenous substrates for target binding, such as endogenous ATP in the context of kinase inhibitors [35].

Not surprisingly, theoretical limits have been placed on the strength of the noncovalent interactions between drugs and their protein targets. For small-molecule ligands whose molecular weight is limited by requirements for oral bioavailability, aqueous solubility, and cellular permeability, the affinity for a protein-binding site is estimated to peak at 10 pM in ideal cases [36,37]. In a widely cited analysis on the maximal affinity of ligands, Kuntz and coworkers reported that covalent inhibitors are common outliers in measurements of drug potencies as a function of heavy-atom count [36]. Houk *et al.* also found that covalent irreversible inhibitors can overcome theoretical limits on potency as a function of molecular size because they are capable of binding to their targets permanently [37].

An additional important advantage of covalent inhibition is the prolonged duration of action that can result from the neutralization of a target under nonequilibrium kinetics. In many cases, the PD of covalent inhibitors can persist even after a drug is cleared by the body or a target organ. For example, a 3-mg dose of rivastigmine, which is a covalent inhibitor of acetylcholinesterase (AChE) for dementia, is sufficient to induce target inhibition for >10 h despite a drug plasma half-life of only 1 h [38]. In this sense, the PK of covalent inhibitors can be uncoupled from associated PD effects. This unique feature of covalent inhibitors might bring certain practical advantages, such an increased scope to advance molecules that have short exposure against a particular target [4]. Indeed, in some cases, it might be beneficial to optimize covalent inhibitors that neutralize a target rapidly while also undergoing fast systemic clearance; such a PK-PD profile of covalent drugs could reduce a patient's drug burden without affecting overall drug efficacy. The prolonged duration of action of covalent drugs could also result in less-frequent drug dosing, thus reducing the risk of idiosyncratic toxicity and potentially improving medication compliance [39]. However, covalent inhibitors against disease targets that undergo rapid protein turnover might not benefit from prolonged duration of action. Indeed, clinical targets that are inhibited covalently often exhibit long protein half-lives that approach 1 day [e.g., epidermal growth factor receptor (EGFR;

16–24 h); hepatitis C virus (HCV) protease (>16 h), and Bruton's tyrosine kinase (BTK; 16–24 h)] [40].

Examples from oncology and virology also suggest that covalent inhibition can work to combat therapy-induced resistance development [4]. Although there are several noncovalent EGFR inhibitors approved in oncology (e.g., gefitinib, erlotinib, and lapatinib), patient populations often develop resistance to these drugs through mutations that increase EGFR kinase activity and affinity for ATP (e.g., L858R and/or T790M) [41,42]. By contrast, covalent EGFR inhibitors, such as afatinib, dacomitinib, neratinib, rociletinib, and AZD9291, have been shown to overcome drug resistance acquired against these earlier inhibitors by leveraging increased biochemical efficiency and nonequilibrium binding [41,43]. In the case of HIV-1 protease inhibitors, the development of drug resistance against reversible inhibitors has been correlated with high k_{off} values [44], suggesting that covalent inhibition, where k_{off} can approach zero, is a suitable strategy for combating resistance [10]. A covalent inhibitor of HCV protease has also been reported that effectively inhibits clinically relevant drug-resistant mutants [45].

Lastly, covalent inhibition may be an underused strategy for addressing challenging targets and 'undruggable' modalities in human disease. In one example, cathepsin K (Cat K) has been characterized as an undruggable cysteine protease target because of its small and solvent-exposed binding site [46]. Despite this perceived challenge, odanacatib is a covalent inhibitor of Cat K that has been advanced to large-scale human trials for treating osteoperosis. It is postulated that other challenging biomolecular targets, such as protein–protein interactions, might similarly be addressed using covalent inhibition. Box 1 summarizes the pros and cons of covalent inhibitors in drug discovery.

Success stories and ongoing clinical studies of covalent drugs

Despite concerns about toxicity, covalent inhibitors have had significant impacts on human health. In this section, I review an assortment of covalent drugs that are being investigated clinically or that are already approved for a range of ailments. Notably, of the 27 approved drugs in 2013, three agents were covalent inhibitors [47].

Oncology indications

Oncology has historically been a rich source of diverse and sometimes unlikely drugs because of the serious medical need of its patients and the often acute administration of therapies (e.g., nitrogen mustards; Fig. 5). As shown graphically, electrophiles used against oncology targets include epoxides, a vinylogous amide, a boronic acid, a β-lactone, and a variety of acrylamides (Figs. 6 and 7).

EGFR

EGFR is a receptor tyrosine kinase implicated in malignancies including non-small cell lung cancer (NSCLC) and glioblastoma [48]. Substituted and terminal acrylamides were found to exhibit an excellent balance of reactivity and selectivity for the Michael reaction with an active site cysteine of EGFR [49]. Although the first covalent kinase inhibitor, canertinib, did not advance past phase II trials, others followed closely and achieved some success. The EGFR inhibitor afatinib [50] was approved in 2013, whereas the EGFR inhibitors rociletinib, dacomitinib, neratinib, and AZD9291 are currently in clinical trials for several cancer indications (Fig. 6) [51].

BTK

BTK is a cytoplasmic kinase that has a role in B cell development and functioning. In addition to hematological malignancies, BTK inhibitors are potential therapies for other cancers and also for autoimmune disorders [52]. The oral inhibitor ibrutinib displays subnanomolar potency against BTK and was approved for B cell malignancies in 2013 [53]. CC-292 (or AVL-292), an additional subnanomolar inhibitor of BTK, is currently in clinical trials for the same indication (Fig. 6) [54].

MEK1

Mitogen-activated protein kinases 1 and 2 (MEK1 and MEK2) play key roles in signal transduction within the Ras-Raf-MEK-ERK1/2 pathway commonly implicated in carcinogenesis [55]. Although a single noncovalent inhibitor of MEK1/2 has been approved in the past decade (trametinib), progress on the discovery of additional MEK1/2 inhibitors for human use has been sluggish because of challenges in achieving acceptable efficacy. E6201 is a low-nanomolar, covalent inhibitor of MEK1 currently in clinical trials for solid tumors and also for psoriasis (Fig. 6). Inspired by the naturally occurring resorcylic acid lactones F152A1 and hypothemycin, E6201 exhibits improved plasma stability and is available for intravenous or topical use [56]. The enone embedded within the macrocyclic ring of E6201 accepts a cysteine nucleophile in the active site of MEK1.

HSP70

Triptolide is a naturally occurring diterpene triepoxide that has attracted attention because of its potent anticancer activity in a variety of preclinical cancer models [57]; triptolide also has anti-inflammatory and immunosuppressive activities (Fig. 6). Although the exact mechanism of action of triptolide has been elusive, evidence suggests that it acts pleiotropically to decrease expression of heat shock proteins, such as HSP70 [57]. Triptolide has been demonstrated to bind covalently to an unidentified protein in nuclear extracts of A549 cells [58], and experimental evidence suggests that all three epoxides are necessary for full anticancer activity. Minnelide is a derivative of triptolide that is water soluble and has recently advanced past the preclinical stage (Fig. 6). Although mechanistic work remains to be reported, minnelide is currently in early clinical trials for patients with refractory gastrointestinal tumors.

PI3K

Phosphatidylinositol 3-kinases (PI3Ks) are a family of signal transduction enzymes that phosphorylate the inositol ring of phosphatidylinositol, with a role in cell proliferation, apoptosis, and other cellular functions. PI3K is altered in many human cancers and has emerged as a promising target in oncology. Wortmannin is a steroidal natural product that is potent but unselective against PI3K, and that acts covalently through an enoate embedded within a furan ring [15]. To address the poor selectivity and biological instability of wortmannin, an analog named PX-866 has been developed as a stable, oral PI3K inhibitor [59]. PX-866 readily reacts with a lysine residue in the catalytic site of PI3K through a vinylogous transamidation reaction, resulting in the irreversible inhibition of this kinase. PX-866 is currently being investigated in clinical trials against several advanced tumors, including glioblastoma and castration-resistant prostate cancer.

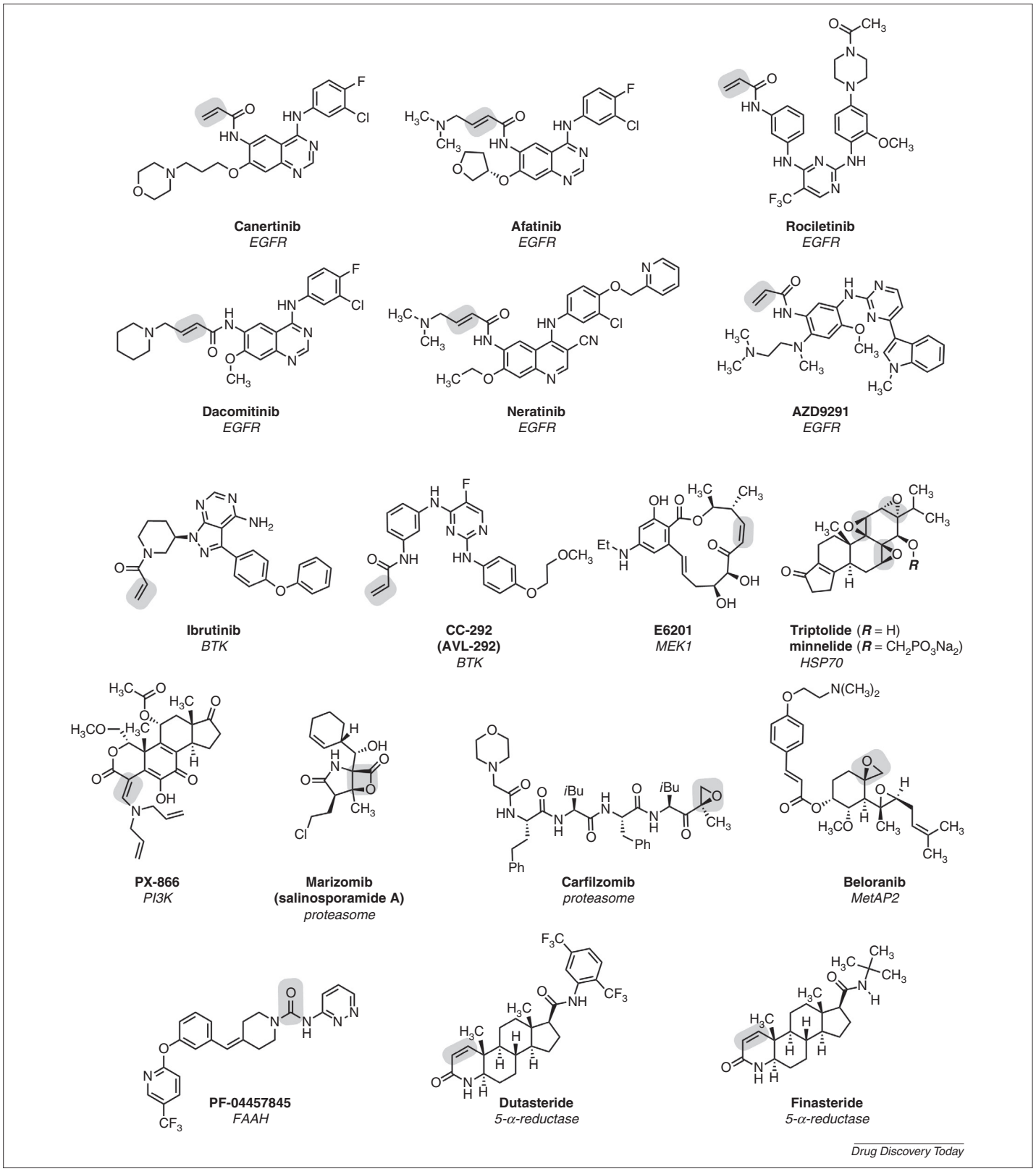

FIGURE 6

Examples of irreversible covalent drugs. Electrophiles are highlighted.

Proteasome

The proteasome is a large protein complex that has a central role in the regulation of cellular function by catalyzing the ATP-dependent degradation of cellular proteins [60]. Bortezomib (Fig. 7) was the first US Food and Drug Administration (FDA)-approved proteasome inhibitor [61], paving the way for the success of other proteasome inhibitors, such as carfilzomib (Fig. 6). Bortezomib and carfilzomib exert their proteasome inhibition though a boronic acid and an

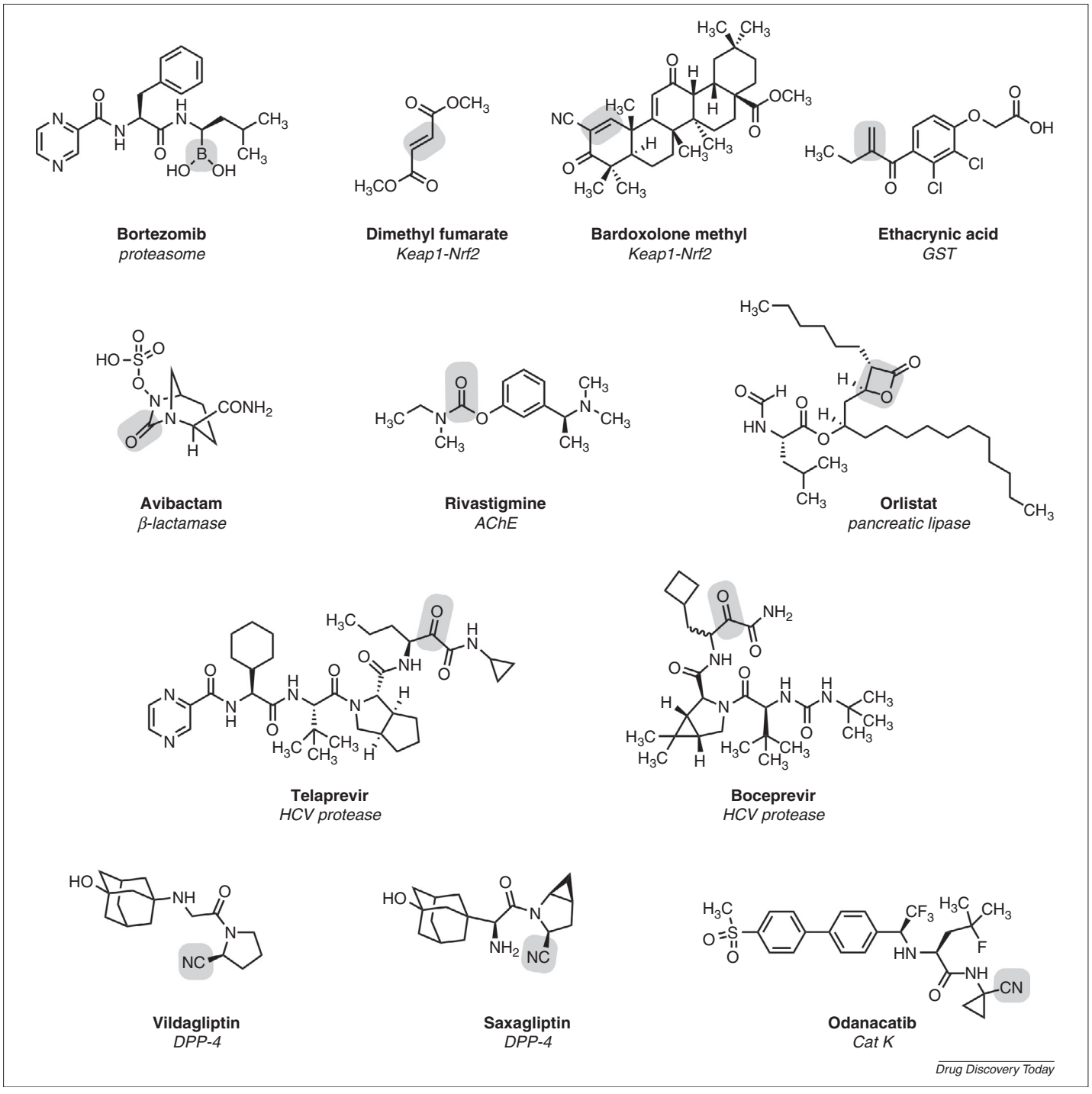

FIGURE 7

Examples of reversible covalent drugs. Electrophiles are highlighted.

epoxide, respectively, which form covalent bonds with an active-site threonine [60]. Marizomib (or salinosporamide A), which is currently in clinical trials, has a β-lactone as its threonine-reactive electrophile against the proteasome (Fig. 6) [60]. Although proteasome inhibitors were initially developed as anti-inflammatory agents, the current proteasome pharmacopeia is directed against various cancers, and patients with multiple myeloma are particularly responsive to these agents. Notably, numerous protease inhibitors trace their roots to natural products. Whereas marizomib is a bona fide natural product, carfilzomib is a derivative of the natural product epoxomicin.

Nononcology indications

Although protein-reactive, covalent drugs are often associated with oncology applications, as many as 80% of approved covalent inhibitors are used in therapeutic areas outside of cancer [4]. As shown in Figs. 6 and 7, electrophiles used for nononcology indications include not only Michael acceptors, but also epoxides,

nitriles, α-ketocarboxamides, ureas, and carbamates. Indications range from obesity to multiple sclerosis.

Keap1–Nrf2 pathway

Nrf2 is a transcription factor that has a role in the cellular response to stress by upregulating genes involved in cytoprotection [9]. Under nonstressed conditions, Nrf2 forms a complex with the scaffolding protein Keap1, signaling the degradation of Nrf2 through nuclear export and proteolysis. In 2013, dimethyl fumarate was approved as an inducer of Nrf2 for multiple sclerosis (Fig. 7) [47]. The metabolite monomethyl fumarate has been shown to alkylate Cys151 of Keap1 [62]. Bardoxolone methyl ester, an α-cyanoenone derivative of oleanolic acid, is also an inducer of Nrf2 through the covalent modification of Cys151 of Keap1 (Fig. 7) [63]. The anti-inflammatory effects of bardoxolone are being evaluated in clinical trials for conditions including pulmonary arterial hypertension and chronic kidney disease [9].

GST

Ethacrynic acid is an approved diuretic drug that inhibits Na^+-K^+-$2Cl^-$ cotransport in the kidneys (Fig. 7) [64]. Although the detailed molecular mechanism of action is not known, ethacrynic acid has been shown to be a potent inhibitor of glutathione *S*-transferase (GST) isozymes [65]. Apart from its diuretic effects, the ability of ethacrynic acid to inhibit GST has implications for combating resistance to certain electrophilic cancer drugs, which are susceptible to inactivation by conjugation to glutathione catalyzed by GST [66]. Ethacrynic acid has been available for use as a diuretic for nearly 50 years.

β-Lactamase

The development of resistance to antibiotics is a significant global challenge [12]. A principal mechanism by which resistance develops to β-lactam antibiotics is through bacterial expression of β-lactamase and inactivation of these antibiotics by β-lactam hydrolysis. Avibactam is a non-β-lactam, covalent inhibitor of β-lactamase that is being evaluated clinically for its ability to restore the efficacy of ceftaroline and ceftazidime in serious infections [67]. The electrophilic urea of avibactam reacts with a serine nucleophile in the active site of β-lactamase.

MetAP2

Methionine aminopeptidases (MetAPs) are metalloproteases that catalyze the cleavage of methionine from the amino terminus of nascent proteins. MetAP2 is suspected to have a role in certain tumors by facilitating angiogenesis and, as such, it has attracted significant clinical interest. During investigations of MetAP2 inhibitors as anticancer agents, it was observed that weight loss occurred even at doses where angiogenesis was not affected [68]. The cellular mechanisms responsible for this effect are not completely understood. Beloranib, an analog of the prototype natural product fumagillin [15], is a di-epoxide covalent inhibitor of MetAP2 that is currently under clinical investigation (Fig. 6) [69]. The less-hindered spiroepoxide of beloranib is responsible for binding to His231 in the active site of METAP2 [68].

Pancreatic lipase

Another pharmacological target against obesity is pancreatic lipase, an enzyme that hydrolyzes triacylglycerol fatty acids. The hydrolysis of dietary fat esters is required by the body to absorb lower-molecular-weight fatty acids. Inhibition of pancreatic and gastric lipase activity results in the passage of unhydrolyzed, intact triacylglycerols through the stool [15]. Orlistat is an oral inhibitor of pancreatic lipase derived from the natural product lipstatin (Fig. 7). The β-lactone of orlistat covalently reacts with a catalytic active-site serine of pancreatic lipase. Orlistat is largely gut localized and exhibits negligible blood plasma levels, a feature that reduces the safety risk of this agent [70].

FAAH

Fatty acid amide hydrolase (FAAH) is an integral membrane protein responsible for hydrolysis of bioactive fatty acid amides, which have a role in pain reception and inflammation. Inhibition of FAAH leads to elevated levels of these fatty acid neurotransmitters. PF-04457845 is an orally available covalent inhibitor of FAAH that is currently in clinical trials to treat chronic pain and other nervous system disorders (Fig. 6) [71]. Evidence suggests that PF-04457845 forms a carbamate linkage with the catalytic nucleophile Ser241 of FAAH, releasing 3-aminopyridazine [72].

AChE

Acetylcholine is a neurotransmitter that stimulates cholinergic receptors at chemical synapses in the central nervous system. Given that patients with Alzheimer's disease (AD) can exhibit decreased levels of these receptors, one pharmacological strategy to combat dementia symptoms of AD involves increasing acetylcholine levels at theses synapses [73]. Rivastigmine is an approved, covalent inhibitor of acetylcholinesterase (AChE), an enzyme that inactivates endogenous acetylcholine (Fig. 7). Once bound to AChE, rivastigmine acylates an active site serine through its phenolic carbamate. Although rivastigmine is cleared relatively quickly, its inhibitory effects on AChE last up to 10 h [38]. Rivastigmine, which is an analog of the natural product physostigmine, is currently used as an oral or transdermal agent to treat dementia in AD and Parkinson's disease.

5-α-Reductase

Steroid 5-α-reductase reduces testosterone to dihydrotestosterone in an NADPH-dependent manner. The biosynthesis of dihydrotestosterone is implicated in the development of large prostates in older men. Finasteride and dutasteride are approved, steroidal enamides that covalently inhibit 5-α-reductase (Fig. 6). The mechanism of action of these agents is unique from other approved Michael acceptors. Both finasteride and dutasteride are mechanism-based inhibitors: once bound to 5-α-reductase, a hydride is donated from bound NADPH in a 1,4-conjugate addition, a step that produces $NADP^+$ and formally an amide enolate of the bound drug [74]. Although the natural testosterone substrate suffers protonation and dissociation from 5-α-reductase, the amide enolate of finasteride and dutasteride attacks enzyme-bound $NADP^+$ to form a covalent bond with this enzyme-linked cofactor. Finasteride and dutasteride are prescribed chronically against benign prostatic hyperplasia.

HCV protease

HCV protease is a drug target for treating HCV infection, a condition that affects 3% of the human population of the world [75]. Inhibition of HCV protease prevents formation of a viral complex that is responsible for RNA synthesis and viral replication. Structure-based drug design has resulted in two approved, covalent therapies against HCV protease. Telaprevir and boceprevir are oral α-ketoamides that bind to HCV protease through a stable hemiketal using Ser-139 in the catalytic site (Fig. 7). This reversible covalent binding against this viral protease has been demonstrated by X-ray crystallography [75].

DPP-4

Dipeptidyl peptidase-4 (DPP-4) is a serine protease involved in the degradation of incretin hormones, such as glucagon-like peptide 1, which regulate insulin and glucagon secretion, and glucogenesis during and after a meal. Covalent DPP-4 inhibitors have been used to manage glucose levels in type 2 diabetes mellitus (T2DM) by enabling patients to endogenously produce their own insulin [76]. Vildagliptin and saxagliptin are approved oral DPP-4 inhibitors for managing blood glucose levels in T2DM (Fig. 7). The common electrophile in these two agents is a nitrile that forms a covalent bond with an active site serine via a Pinner reaction.

Cat K

Cathepsin K (Cat K) is a cysteine protease that has a role in the degradation of collagen, which is a non-mineral component of bone [77]. Given that collagen degradation is implicated in mammalian bone resorption, Cat K is an attractive target for treating osteoporosis-related bone loss. Odanacatib is a Cat K inhibitor currently in large-scale clinical trials for reducing bone fractures in older women [78]. Odanacatib has an electrophilic nitrile that acts covalently on Cat K (Fig. 7). Whereas the nitrile-containing vildagliptin and saxagliptin react with a serine in the active site of DPP-4 to generate imidates, the nitrile of odanacatib reacts with a cysteine in the Cat-K active site to generate a thioimidate intermediate. Notably, Cat K has been identified as a borderline undruggable enzyme because of its small and solvent-exposed binding site [46]. Despite this, odanacatib is a potent inhibitor of Cat K and, as a result of its long half-life and prolonged inhibiton, it is being investigated as a once-weekly oral osteoperosis agent.

Comments on reversible covalent inhibitors

Whereas many acrylamide- and epoxide-containing drugs (e.g., afatinib and carfilzomib) form permanent bonds with their biological targets, other electrophiles used in drugs, such as nitriles (e.g., vildagliptin), electron-deficient ketones (e.g., boceprevir), or enoates (e.g., dimethyl fumarate), have been found to form reversible covalent bonds with their respective targets (Fig. 7). In practice, it is often not possible to make binary assessments of the reversibility of covalent inhibitors, and rather reversibility is better interpreted along a spectrum of drug residence time. In some cases, the binding of covalent reversible inhibitors is short lived, but in other cases even reversible binding can be durable if assisted by stabilizing noncovalent interactions. While an X-ray of bardoxolone bound to Keap1 has recently been reported [63], studies using low-molecular-weight thiols indicate that bardoxolone thioethers can rapidly undergo retro-Michael addition [79]. As such, bardoxolone has been widely recognized as a reversible covalent inhibitor that rapidly dissociates from its target [8,9]. By contrast, the reversible covalent inhibitor rivastigmine, which forms a carbamate linkage in the active site of AChE, is known to dissociate relatively slowly, leading to complete restoration of enzyme activity over 8 h [38]. Johanasson referred to the latter kind of reversible covalent binding as 'dock-and-lock binding' [8]. Furthermore, a covalent inhibitor can be considered irreversible in one context but also reversible in another context. Neratinib not only inhibits EGFR irreversibly, but has also been found to bind covalently at Lys190 in human plasma albumin in a reversible manner [80]. Reversible covalent inhibitors are thought to have a lower risk of toxicity because potential drug–protein adducts based on these agents might not reach sufficient levels to induce a host immune response. The Taunton laboratory has systematized this concept with the development of libraries of α-cyanoacrylamides that reversibly bind to thiol nucleophiles in targets such as the kinase RSK2 [17,81]. A range of diverse reversible covalent inhibitors is illustrated in Fig. 7.

Concluding remarks

Covalent inhibitors have enjoyed a long history in drug discovery, beginning with aspirin during the late 19th century and culminating with a recent surge of rationally engineered kinase inhibitors entering clinical studies. Until recently, covalent drugs were not designed deliberately, with their covalent binding modes being elucidated only after development (in the case of aspirin, almost a century after its synthesis). Lessons from natural products have led to the development of many approved and clinically investigated drugs. The influence of natural products on covalent drug development is illustrated through a comparison of physicochemical properties of the 36 covalent inhibitors (reviewed here) with a diverse set of 60 natural products and also with 36 top-selling noncovalent, brand name drugs [82]. For nine properties analyzed (molecular weight, nitrogen and oxygen count, cLogP, H-bond donors and H-bond acceptors, rotatable bonds, polar surface area, and stereocenters), one finds that clinically relevant covalent inhibitors fall closer to natural products than do conventional drugs (Fig. 8). Although only a modestly sized data set, it is easy to see the connections of the covalent inhibitors described here with natural products, as reflected in the structures of β-lactam antibiotics, fosfomycin, E6201, minnelide, PX-866, orlistat, beloranib, rivastigmine, salinosporamide A, and carfilzomib.

Indeed, literature citations concerning covalent inhibitors seem to be rapidly increasing over time (Fig. 1) and as many as three covalent inhibitors were approved in 2013 alone. Several reasons might account for this continued interest in covalent inhibitors. First, the increased biochemical efficiency of covalent inhibitors (as a function of molecular size) may provide opportunities to reduce the dose of a drug, which has important implications for improving therapeutic windows and for decreasing the risk of idiosyncratic toxicity that is difficult to predict preclinically. Second, the prolonged duration of action of covalent inhibitors might also provide for less-frequent drug dosing and might potentially improve patient compliance [39]. Finally, covalent inhibition might mitigate the emergence of therapy-induced drug resistance in therapeutic areas such as cancer and infection, while also presenting attractive opportunities to address challenging binding sites for targets such as protein–protein interactions (Box 1).

Despite these attractive features, the liabilities of covalent inhibitors resulting from potential nonspecific reactivity are still widely appreciated and, as a result, electrophilic functional groups are frequently excluded from screening collections and from discovery campaigns all together. Avoiding promiscuous alkylation of macromolecules by drugs is indeed a central goal of covalent drug discovery, but it is interesting that many approved and safely prescribed drugs are known to form covalent adducts with thiols *in vitro* [83]. The alkylation of human plasma protein by the kinase inhibitor neratinib suggests that nonspecific protein binding does not in principle prevent clinical success [80]. Discovery teams that

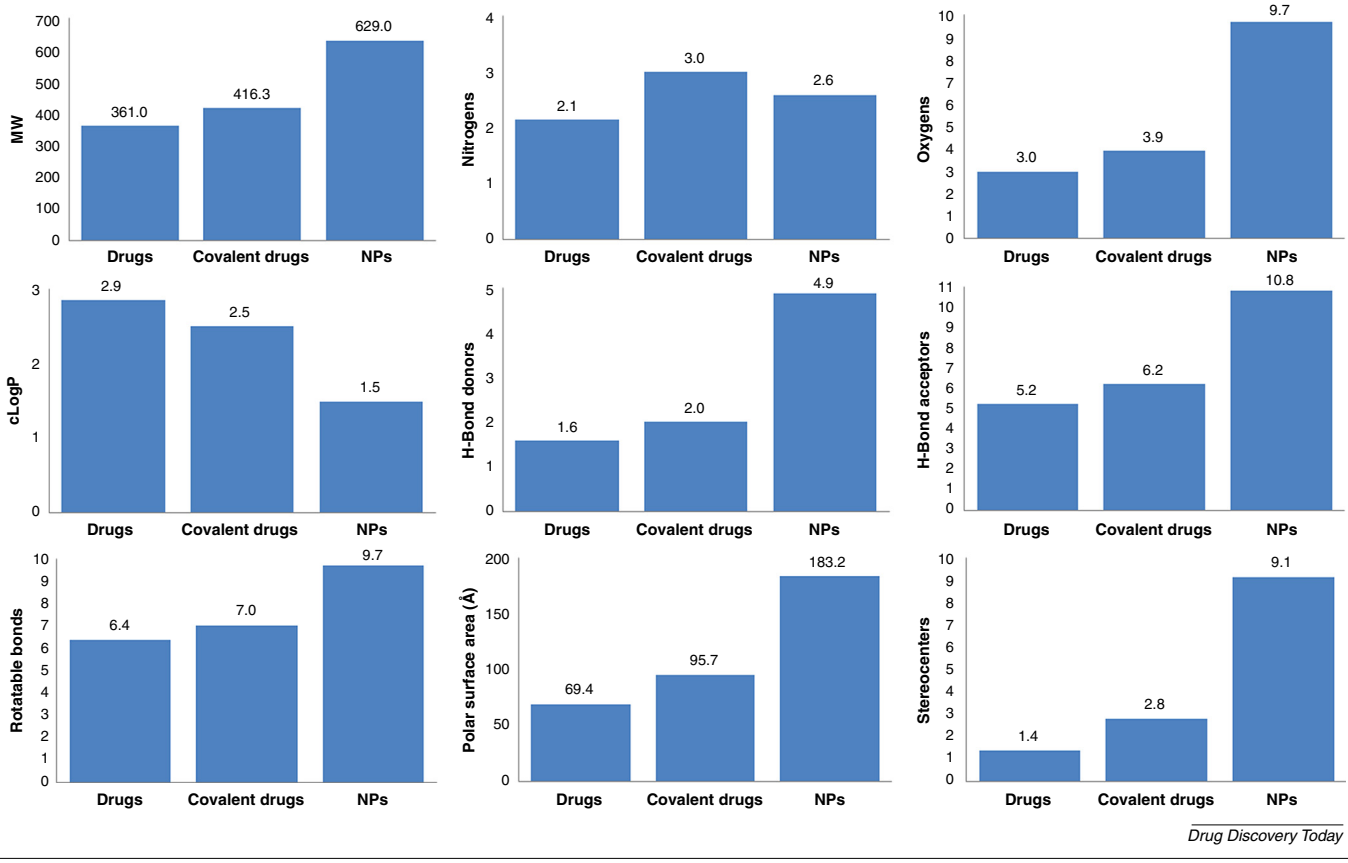

FIGURE 8

Comparison of nine physicochemical properties (molecular weight, nitrogen and oxygen count, cLogP, H-bond donors and H-bond acceptors, rotatable bonds, polar surface area, and stereocenters) of 36 covalent inhibitors covered in this review with 60 diverse natural products (NPs) and 36 top-selling brand name (noncovalent) drugs described in [82].

set out to work on covalent inhibitors must balance a tolerance for preclinical adduct formation against issues relating to the projected dose of a drug, the medical need of a patient population, and the benefits associated with covalent drugs, such as increased potency and prolonged duration of action. Thus, a multipronged approach to assessing and mitigating covalent toxicity risk is suggested here and has been advocated by others [7,40,84]. Literature reports are being disclosed with increasing frequency to describe the range of biological reactivity of diverse electrophiles in compound collections [85–90]. If this is any indication of the future clinical landscape of small-molecule drugs, more covalent inhibitors may be expected to enter clinical development in the coming years.

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