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Review
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Successful strategies in the discovery of small-molecule epigenetic modulators with anticancer potential

As a class, epigenetic enzymes have been identified as clear targets for cancer therapeutics based on their broad hyperactivity in solid and hematological malignancies. The search for effective inhibitors of histone writers and of histone erasers has been a focus of drug discovery efforts both in academic and pharmaceutical laboratories and has led to the identification of some promising leads. This review focuses on the discovery strategies and preclinical evaluation studies of a subset of the more advanced compounds that target histone writers or histone erasers. The specificity and anticancer potential of these small molecules is discussed within the context of their development pipeline.

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Epigenetic mechanisms play key roles in the regulation of cell physiology in normal and disease states. DNA and histone modifications alter the structure of chromatin affecting DNA accessibility and ultimately DNA-based processes, such as transcription, DNA repair and replication [1–3]. In particular, regulatory histone post-translational modifications (HPTMs) are in general reversible marks that mainly target the exposed N-terminal tails of these proteins. Many distinct types of chemical histone modifications have been described of which histone phosphorylation, acetylation, methylation and ubiquitination are the best understood [3,4]. Each specific PTM is dynamically regulated by two sets of enzymes: writers that add the marks to the histone, and erasers that remove the PTM. In addition, a set of specialized proteins with recognition domains, known as readers, use these PTMs as docking sites to direct downstream events on chromatin [5].

Research over the last two decades has revealed that epigenetic reprogramming can allow the cell to acquire features characteristic of cancer [6]. Indeed, when defining the molecular mechanisms involved in neoplastic transformation, recurrent mutations and misregulation of histone- and DNA-modifying enzymes and nucleosome-remodeling complexes have been described for multiple solid and hematological malignancies [7]. This emerging knowledge in conjunction with new tools to study epigenetic pathways has pointed to the enzymes and proteins involved in epigenetic pathways as therapeutic targets [8,9]. Starting with the approval of two DNA methyltransferase inhibitors and followed by two histone deacetylases inhibitors, the first generation of epigenetic-based drugs have shown some success in the clinic, limited to specific hematological cancers [10–13]. Despite advances in the understanding of the human epigenome, the next generation of drugs that target it are still in the development stage. Counterintuitively, inhibition of both writers and erasers for a PTM such as histone methylation can result in antitumoral effects and are therefore of interest. Different tumoral cells can become addicted to different epigenetic enzymes in some contexts, such as in the presence of activating genetic mutations in a writer of a repressive mark and/or amplification of an eraser of a mark. Cancer can deregulate the entire regulatory pathway for any histone PTM and this phenomenon allows for antitumoral effects through inhibiting either a writer or a eraser

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Key terms

Histone post-translational modifications (HPTMs): Regulatory post-translational modifications of histones including acetylation, methylation, phosphorylation, ubiquitination, etc.

Writers: Enzymes that add regulatory post-translational modifications to histone tails.

Erasers: Enzymes that delete post-translational modifications from histone tails.

High-throughput screening (HTS): High-throughput screening assays used in drug discovery to identify small molecules of interest.

in specific cases. In this review, we will focus on recent strategies used by the academic and pharmaceutical communities to identify and develop a subset of effective inhibitors of writers and erasers of histone PTMs, with anticancer properties. While not comprehensive in scope, this review will highlight some of the so far successful discovery-to-application drug development pipelines that have reached animal studies. These inhibitors, among others, represent the next generation of epigenetic compounds developed by combining knowledge of chromatin-associated proteins in cancer and advances in technologies of molecular, cellular and structural biology (Figure 1).

Targeting histone writers

In the 1960s it was proposed that histone PTMs and transcriptional regulation could be related: however, it was not until 1996 that the first histone acetyltransferase was described and a few years later the first histone methyltransferase [14,15]. Since then, multiple families of diverse histone writers have been described. Histone writers catalyze the addition of PTMs on histones in a dynamic response to different stimuli and as a class are highly deregulated in many types of cancer including non-small-cell lung cancer, prostate cancer and acute myeloid leukemia [16–18]. Examples of successful inhibitor discovery efforts are highlighted below and summarized in Table 1.

Inhibition of histone acetyl transferases

Acetylation of the amino group of specific lysines of histone tails changes the chromatin conformation to an open state by affecting the electrostatic interactions between the DNA negative charge and the lysine positive charge [49]. In addition, this allows the binding of transcription factors that contain reader domains for histone acetylation such as bromodomains and tandem plant homeodomain (PHD) [50,51]. Histone acetyl transferases (HATs)/lysine acetyl transferases (KATs) include several families: the GNAP (tGcn5, PCAF and ELP3), the P300/CBP family, and the MYST family (TIP60 and MYST 1–4) which are localized in the nucleus [1,52–53]. The different cellular acetyl transferases share low sequence and structural homology with only a conserved acetyl CoA binding pocket making the development of specific inhibitors challenging [54,55].

HATs contribute to the oncogenic process through association with viral oncoproteins, through chromosomal translocations, local mutations and altered expression in different cancer types leading to transcriptional deregulation [14,56–60]. HAT inhibitors include bisubstrate inhibitors, natural products derivatives and synthetic small molecules. Bisubstrate inhibitors, such as Lys-coenzyme A conjugates and histone 3-coenzyme A conjugates were among the first to be described although with poor results *in vivo* due to low permeability and the lack of metabolic stability [55,61–62]. In contrast, natural products and their derivatives have shown promising anticancer properties yet pleiotropic effects make it difficult to clarify their actual mechanism of action [19,55]. A few compounds have been described with high specificity for histone acetyl transferases and anticancer activity.

p300 and its paralog CBP acetylate several histone residues including K14 and K18 of H3 and K5 and K8 of H4 and its mutations and deregulation have been associated with different types of cancer including leukemia, colon cancer and breast cancer [20]. Bowers *et al.* used virtual ligand screening to test a library of 500,000 commercially available compounds and found a small molecule that binds into the Lys-CoA p300/CBP binding pocket [21]. C646, a p300/CBP linear competitive inhibitor of Acetyl CoA, was discovered using this *in silico* approach [21]. Further analysis and experimental validation showed C646 is selective for p300/CBP in comparison with other HATs and reduces the levels of histone acetylation in mouse fibroblast (C3H 10T1/2), melanoma (WM983A) and leukemia (Kasumi-1) cell lines [21,22]. C646 also demonstrated antigrowth effects in melanoma, lung cancer and prostate cancer cell lines *in vitro* [21–23,63]. One report has demonstrated that C646 can have anticancer activity *in vivo*. Gao and colleagues showed in a mouse model of acute myeloid leukemia that treatment with C646 can increase the survival of mice by suppressing *in vivo* growth of transplanted leukemia blasts [22]. Similarly, a virtual screening of the National Cancer Institute (NCI) compound collection and several commercial compound libraries, allowed the finding of PU139 and PU141, two inhibitors that dock into the PCAF catalytic domain [24]. Validation experiments showed that PU139 is a potent pan-HAT inhibitor, while PU141 is a CBP/p300-selective inhibitor [24,64]. Both

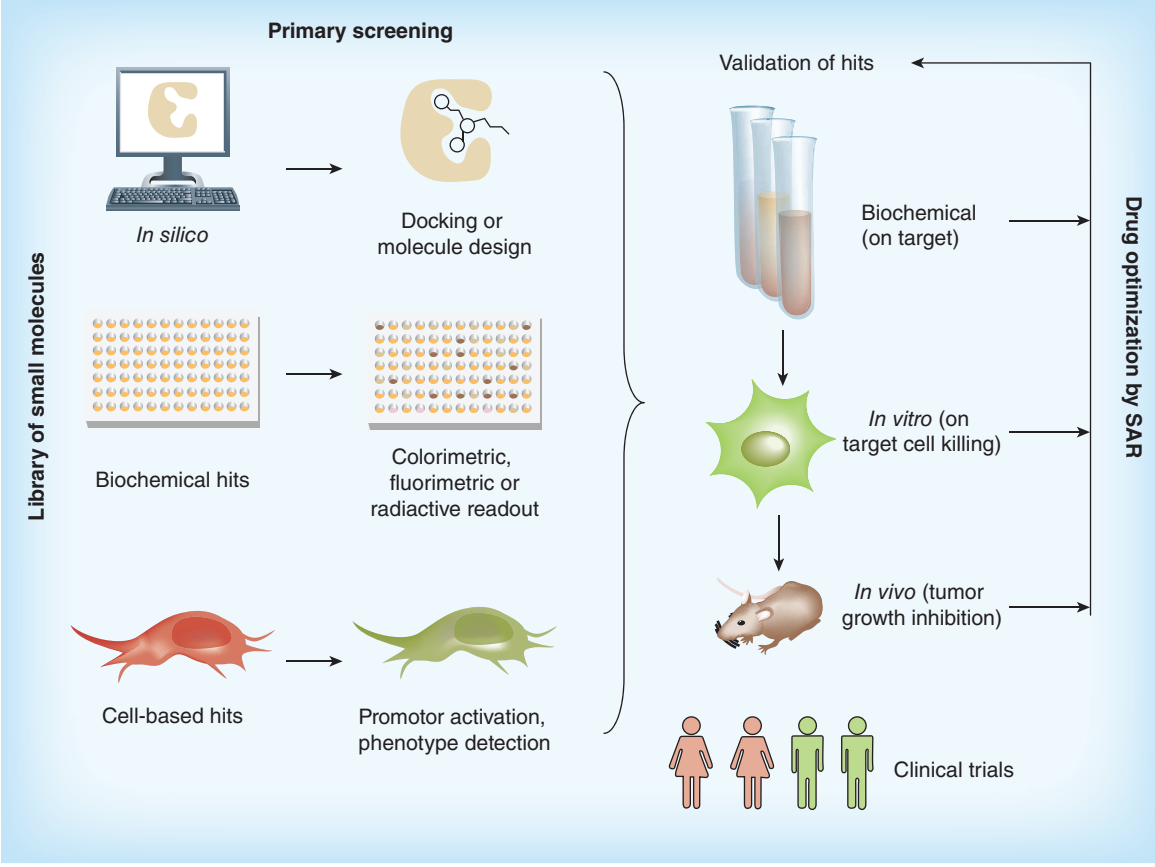

Figure 1. Successful strategies used in the discovery and development of epigenetic inhibitors with anticancer activity. Small-molecule candidates are identified using *in silico*/structural, biochemical, or cell-based high-throughput screening approaches. Hits are validated using biochemical assays to determine their selectively and potency. Top hits are then tested in select cancer cell lines to determine their on target activity and potency, followed by mouse cancer models for their antitumor, pharmacokinetic and toxicity properties. SAR studies guide optimization and the development of second-generation inhibitors with improved properties. Small molecules with the best profiles and targeted indications may enter human clinical trials.
SAR: Structure–activity relationship.

compounds reduce the acetylation levels of H3 and H4 and show antiproliferative properties in neuroblastoma and colon carcinoma cells. The anticancer properties of these HAT inhibitors were verified in a neuroblastoma xenograft and the reduction in histone acetylation in healthy mice [64].

Tip60 catalyzes the acetylation of H2AK5, H3K14 and K5 and K8 of H4 and can function as an oncogene. In prostate cancer TIP60 has been associated with the development of resistance to chemotherapy [25]. OXA-10 was discovered using a biochemical high-throughput screen (HTS) for TIP60 inhibitors based on the ALPHA assay and confirmed by DELFIA immunoassay, over a structurally diverse collection of 80,000 compounds [25]. However, further analysis indicated that it doesn't have selectivity over p300. Analysis of analogs of OXA-10 allowed the discovery of an isothiazolone compound, NU9056, a TIP60 selective inhibitor over PCAF, p300 and GCN5. *In vitro* experiments in LNCaP cells showed that NU9056 is able to reduce the levels of H3 and H4 acetylation and induce apoptosis [25].

A library of 622,079 small molecules was tested for their cytotoxicity to the MDA-MB-231 cell line but not to human mammary epithelial cells. In a second step of selection the L002 compound was identified as a p300 inhibitor in a biochemical assay. L002 is able to inhibit CBP, PCAF and GCN5 but not other HATs [26]. In addition, L002 is able to decrease the levels of H3 and H4 acetylation in MDA-MB-231 cells and has cytotoxic effects against triple negative breast cancer and hematologic cancer cell lines. Antitumor activity and reduction of histone acetylation was reported in mice bearing MDA-MB-468 xenografts [26].

Inhibition of histone methyltransferases

Histone methylation was reported for the first time in 1964 and it was considered as an irreversible PTM until 2004 when the first histone lysine demethylase was discovered [65,66]. Now it is known that histone

Table 1. Selective small-molecule inhibitors of histone acetyl transferases, lysine methyltransferases and protein arginine methyltransferases.

Compound	Target	Discovering strategy	Ref.
HAT inhibitors			
C646	p300/CBP	*In silico* screening	[19–22]
PU139	Pan HATs inhibitor	*In silico* screening	[23,24]
PU141	p300/CBP	*In silico* screening	[23,24]
OXA-10	TIP60	Biochemical HTS	[25]
NU9056	TIP60	Chemical optimization of OXA-10	[25]
L002	p300/CBP, PCAF, GCN5	Cell-based and biochemical HTS	[26]
KMT inhibitors			
BIX-01294	G9a, GLP	*In silico* screening and biochemical HTS	[27,28]
UNC0642	G9a, GLP	SAR of BIX-01294	[29]
EPZ04777	DOT1L	Structure-based design	[30,31]
SGC0946	DOT1L	SAR analysis of EPZ0477	[32]
EPZ05676	DOT1L	SAR analysis of EPZ0477	[33]
MI-1	MLL–menin interaction	Biochemical HTS	[34]
MI-2	MLL–menin interaction	Chemical optimization of MI-1	[34]
MI-3	MLL–menin interaction	Chemical optimization of MI-1	[34]
MI-2-2	MLL–menin interaction	Chemical optimization of MI-2	[35]
MI-463	MLL–WDR5 interaction	Chemical optimization of MI-2-2	[36]
MI-503	DOT1L	Chemical optimization of MI-2-2	[37]
MM-102	MLL–WDR5 interaction	Structure-based design	[36]
MM-401	DOT1L	SAR analysis of MM-102	[37]
EPZ05687	EZH2	Biochemical HTS	[38]
GSK-A	EZH2	Biochemical HTS	[39]
GSK-126	EZH2	SAR analysis of GSK-A	[39]
E1	EZH2	Biochemical HTS	[40]
UNC01999	EZH2	SAR analysis of EPZ05687 and GSK-126	[41]
EPZ06438	EZH2	SAR analysis of EPZ05687	[42,43]
Compound 3	EZH2	Biochemical HTS	[44]
Compound 44	EZH2	Chemical optimization of compound 3	[44]
CPI-360	EZH2	Chemical optimization of compound 44	[45]
CPI-169	EZH2	Chemical optimization of CPI-169	[45]
CPI-1205	EZH2	ND	[46]
PRMT inhibitors			
EPZ015666	PRMT5	Biochemical HTS	[47]
SGC707	PRMT3	Structure-based design	[48]

HAT: Histone acetyl transferase; HTS: High-throughput screening; KMT: Lysine methyltransferase; ND: Not described; PRMT: Protein arginine methyltransferase; SAR: Structure–activity relationship.

methylation is a dynamic process that involves a balance between lysine or arginine methylases/demethylases [67–69]. Histone methylation, in contrast to acetylation, does not directly change the charge of histones. Instead, methylation facilitates or reduces the accessibility of proteins that regulate the different DNA-based events depending on the specific histone residue that is modified [67]. At least 28 histone lysine meth-

yltransferases (KMTs) have been described. They are divided into eight classes and all together catalyze with high selectivity the methylation of 17 different lysine residues [70–72]. With the exception of DOT1L, which lacks the domain, the KMTs transfer a methyl group from s-adenosylmethionine (SAM) using a highly conserved SET domain. Protein arginine methyltransferases (PRMTs) are able to methylate seven different histone arginine residues: Type I PRMTs monomethylate arginine and generate asymmetric dimethylarginine; Type II PRMTs produce monomethylarginine and symmetric dimethylarginine [73]. Several reports have related aberrant histone methylation during the carcinogenesis process [74,75]. Subsequently, in recent years, great effort has been invested in the development of histone methyltransferase (HMT) inhibitors with anticancer properties.

Inhibition of KMTs

G9a and GLP are two H3K9 methyltransferases that share 80% sequence identity [76]. G9a is upregulated in leukemia and solid tumors and its knockdown reduces cell growth in some cases [77–81]. BIX-01294, one of the first selective small-molecule inhibitor of KMTs, was identified from the Boehringer Ingelheim chemical compound library using a first step of chemoinformatics/structural prediction for KMT inhibitors followed by a second step of HTS based on the DELFIA immunoassay to detect generation of K9me2 on an H3 peptide [27]. Additional validation experiments showed that BIX-01294 is highly selective for G9a and GLP and binds to their H3 peptide-binding groove [27,28]. *In vitro* studies demonstrated that BIX-01294 reduces H3K9me2 global levels and H3K9me2 marks in promoters of several G9a target genes [27]. Unfortunately, the separation within its KMTs inhibition potency and general cell toxicity was low making its potential use as anticancer therapy challenging. Further optimization of BIX-01294, using structure–activity relationship (SAR) analysis, allowed the discovery of a new set of G9a/GLP inhibitors (UNC0224, UNC0321, UNC0631, UNC0638, UNC0646 and E72) that display selectivity in biochemical and cell-based assays [82–85]. UNC0642 showed good bioavailability in mice and a robust potential therapeutic window [29].

MLL catalyzes mono-, di- and tri-methylation of H3K4 and its deregulation plays a key role in the leukemogenesis process in both acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) [86]. In particular, translocation of MLL results in the gained ability to recruit DOT1L, an H3K79 methyltransferase. Loci targeted hypermethylation of H3K4 and H3K79 results in the upregulation of genes that drive leukemogenesis [32]. A design based on the structure of the active site of DOT1L and its interaction with SAM was used by Daigle *et al.* to discover EPZ04777 [30]. This compound was among the first KMT SAM competitive inhibitors. Despite the high similarity with SAM, EPZ04777 displays more than 1000-fold selectivity for DOT1L over other methyltransferases [30]. As for BIX-01294, further optimization by structural analysis led to a series of compounds with improved potency, selectivity and pharmacokinetic properties including SGC0946 and EPZ05676 [32,33]. In AML cell lines with rearrangements in the *MLL* gene, this series of compounds demonstrated a selective reduction in the levels of mono- and di-H3K79 and effective cell killing [30,32–33]. The effect of these compounds on wt AML cell lines was reduced [30,32–33]. An exception of this selectivity for MLL rearranged AML was in AML cell lines with IDH1 and IDH2 mutations which showed good response to EPZ04777 treatment [31]. It should be noted that EPZ05676 is able to achieve complete tumor regression in a subcutaneous model of AML with MLL rearrangement (MV4–11 xenograft). Furthermore, xenografts of treated mice had low H3K79me2 levels indicating DOT1L inhibition [32]. These results led Epizyme to initiate Phase I clinical trials using EPZ05676 to treat AML patients [87]. This is a significant advance in the use of KMT inhibitors as anticancer therapies constituting the first human study of a methyltransferase inhibitor.

A different set of epigenetic inhibitors with antitumor properties against leukemia was developed targeting the MLL H3K4 methyltransferase activity. These inhibitors target the interaction of MLL with proteins such as menin or WDR5 that are essential for its leukemogenic activity [88,89]. Shi *et al.* reported that MI-2 and MI-3 small molecules interact with menin, a key cofactor that binds to both wild type and fusion MLL [34]. These compounds were discovery by optimization of a hit (MI-1) identified in a collection of 49,000 small molecules by HTS based on a fluorescence polarization (FP) assay and validated by NMR. The FP assay used a fluorescein-labeled MLL-derived peptide that contained the high-affinity menin-binding motif (MBM1) of MLL and monitored changes in fluorescence polarization in the presence of compounds. These compounds and a second-generation compound called MI-2–2, showed on target activity by reducing the levels of *HOXA9* and *MEIS1*, two MLL target genes. In addition, they reported anti-

Key term

Structure–activity relationship (SAR): Structure–activity relationship studies utilized in optimization of compound properties.

proliferative, proapoptotic activity and induction of differentiation of leukemia cells harboring MLL translocations [34,35].

In addition, MM-102, a linear peptidomimetic that interferes with the interaction between MLL1 and WDR5, has been developed based in the crystal structure of this complex [36]. This peptidomimetic reduces methylation catalyzed by MLL1 *in vitro* and shows on target activity in cells by the reduction *HOXA9* and *MEIS1*. MM-102 also inhibits cell growth and induces apoptosis in leukemia cells bearing MLL rearrangements [36]. This work allowed the later discovery of MM-401, a new inhibitor of the wild type MLL1–WDR5 interaction [37]. This peptide has improved potency in cell culture reducing the H3K4 methylation levels on MLL1 target promoters. In addition, MM-401 is able to induce cell cycle arrest, apoptosis and myeloid differentiation in leukemia cells without affecting normal bone marrow cells [37]. Recently, MI-463 and MI-503, second-generation inhibitors of the menin–MLL interaction, were developed by optimization of MI2–2 [90]. These compounds have improved pharmacokinetic profiles, high oral bioavailability and are effective against MLL leukemia in a xenograft model without affecting normal hematopoiesis [90].

EZH2, an H3K27 methyltransferase, is upregulated and alters gene expression in different types of hematological and solid cancers [91]. A biochemical HTS of a 175,000 compound library and successive optimization, allowed the discovery of EPZ05687, the first EZH2 inhibitor [38]. This compound was the first of a series that included GSK-A, GSK-126, EI1, UNC1999 and EPZ06438 that share a pyridone and indole/indazole core [39–41,92]. EPZ05687 is a SAM competitive inhibitor with 50-fold selectivity for EZH2 over EZH1 and 500-fold selectivity over other methyltransferases. *In vitro* validation showed that EPZ05687 reduced H3K27 methylation levels in lymphoma cells and caused a small increase in H3K27 acetylation. Interestingly, EPZ05687 selectively induced apoptosis in cell lines carrying EZH2 catalytic domain point mutations [38]. Like their predecessor, the GSK-A and EI1 compounds were discovered using a biochemical high-throughput screen measuring the incorporation of radioactive methyl groups to a biotinylated H3K27 peptide by the EZH2 containing PRC2 complex. GSK-126 was developed after the optimization of GSK-A, a hit identified among 2 million compounds in the GlaxoSmith-Kline collection [39,92]. Both GSK-126 and El1 show similar potency against mutant and wild types EZH2 and high selectivity over EZH1 and other methyltransferases [39,40]. As follow up of the previous work, Konze *et al.* developed UNC1999, the first orally bioavailable inhibitor of both EZH2 and EZH1. The development of this compound was performed by docking EPZ05687 into an EZH2 homology model based on the structure of GLP and using the structural features of EPZ5687 and GSK-126 [41]. EI1 and UNC1999 are SAM competitive inhibitors that are able to reduce H3K27 methylation levels in cells and induce selective apoptosis of cell lines carrying EZH2 mutants [39–41]. GSK-126 has been evaluated in B-cell lymphoma xenografts carrying an EZH2 point mutant (A677G or Y41N) and showed potent antitumor effects in this model [39]. Most recently, Knutson *et al.* developed EPZ06438, an EPZ05687 derivate, through iterative medicinal chemistry [42]. This new compound has similar mechanism of action and selectivity for EZH2 but EPZ06438 has higher cellular activity and improved pharmacokinetic properties. *In vivo* studies demonstrated that EPZ06438 treatment of mice bearing SMARCB1-deleted malignant rhabdoid xenografts causes complete tumor regression and reduction in the H3K27me3 levels [42]. Similar results have been recently reported in an EZH2-mutant non-Hodgkin lymphoma (NHL) model leading EPZ06438 to enter human clinical trials for lymphoma and solid tumors with high levels of H3K27me3 [43]. Garapaty-Rao *et al.* found a structurally distinct novel series of EZH-2 inhibitors through a similar biochemical HTS of 150,000 small molecules from the unbiased Constellation compound collection [44]. In particular, compound 3 showed high selectivity for EZH2 over WHSC1, SETD7, DOT1L, EHMT2 and SETD8, and reduced cell viability and H3K27me3 in B-cell-like diffuse large B-cell lymphoma (GCB-DLBCL) [44]. Further efforts led to the identification of compound 44, a SAM competitor, that reduces global levels of H3K27me3 and induces growth arrest in KARPAS-422 lymphoma cells [93]. A hybrid compound series of these previous compounds with CPI-905, a weak inhibitor with a pyridone head group, was developed by Bradley *et al.* In particular, CPI-360 and CPI-169 are able to inhibit EZH2 at subnanomolar doses [45]. Both inhibitors are selective for EZH2 over a large panel of KMTs, PRMTs and DNA methyltransferases. In addition, CPI-360 and CPI-169 treatment reduced cell viability in a large NHL cell line panel. Moreover, mice bearing GCB-DLBCL xenografts treated with CPI-360 or CPI-169 showed reduction in the tumor volume and in the intratumoral levels of H3K27me3 [45]. Recently, Constellation Pharmaceuticals started a Phase I clinical trial for the treatment of B-Cell Lymphomas with CPI-1205, a new-generation EZH2 inhibitor from this series [87].

Inhibition of PRMTs

Similar to lysine methylation, aberrant arginine methylation pathways are active in cancer. In particular, PRMT5, which catalyzes arginine monomethylation and symmetrical dimethylation, is upregulated in lymphomas, lung cancer, breast cancer and colorectal cancer [47]. Although highly potent and selective KMT inhibitors with cellular activity and anticancer properties have been reported, efforts in development of PRMT inhibitors have been less successful. A HTS based in an ELISA assay of a small library (9000 compounds) led Cheng *et al.* to the discovery of AMI-1, a pan inhibitor of PRMTs [94]. Since then, HTS based on enzymatic *in vitro* activity, virtual screening, *in silico* SAR analysis and further optimization studies were used to identify several inhibitors that target PRMT1, PRMT3 and PRMT4 [75,95–104]. Unfortunately, most of them show low potency (IC_{50} in the μM range), low selectivity or inadequate validation in cell activity studies. Per our knowledge only EPZ015666, a PRMT5 inhibitor, has shown anticancer effects in cells and *in vivo* [47]. *In vitro* studies showed it has 2500-fold selectivity for PRMT5 over other PRMTs. Experiments in mantle cell lymphoma cells (MCL) showed EPZ015666 on target activity and its correlation with antiproliferative effects. *In vivo* studies demonstrated that the compound has good pharmacokinetic properties and oral bioavailability resulting in an antitumor dose response against MLC xenografts. Furthermore, a reduction in the levels of symmetrically dimethylated PRMT5 substrates were observed in the tumors, suggesting on-target activity *in vivo* [47]. SGC707,a PRMT3 inhibitor recently developed by structure-based design and synthesis, should also be mentioned [48]. This compound is an allosteric inhibitor that shows selectivity for PRMT3 against other PRMTs and more than 250 non-epigenetic targets. In addition, SGC707 is able to inhibit PRMT3 in cell culture and is bioavailable after its administration in mice. Further cell and *in vivo* studies are necessary to test its anticancer properties [48].

Targeting histone erasers

From the therapeutic standpoint, the most widely studied erasers of histone marks include histone deacetylases and lysine demethylases. These enzymes have been found to be either genetically amplified or overexpressed in many tumors, thereby making them potential targets for anticancer therapy. Here, we review a subset of inhibitors that have made it from discovery to preclinical evaluation. These small molecules and their targets are summarized in Table 2.

Inhibition of histone deacetylases

Histone deacetylases (HDACs) remove acetylation marks deposited by HATs by catalyzing the hydrolysis of N-acetyl lysine residues and thus lead to gene silencing. In cancers, aberrant recruitment of HDACs to promoters due to chromosomal translocations, fusion proteins and/or HDAC overexpression causes silencing of tumor suppressor genes. Many studies have implicated the involvement of histone deacetylases in cancer progression, tumor cell survival, metastasis and chemoresistance [138–141]. HDACs can be categorized into class I, II and IV, all of which are zinc dependent enzymes, and NAD^+-dependent sirtuins or Class III HDACs that do not rely on zinc. Thus, most known HDAC inhibitors target this zinc dependence and possess chelating groups. Over the past decade, extensive efforts have been put towards development of such small-molecule inhibitors of histone deacetylases. Since inhibitors of zinc-dependent HDACs are established and have been extensively covered in multiple other reports [105,142–143], here we will only briefly review common classes of HDAC inhibitors and strategies used in their identification.

The pharmacophore of most HDAC inhibitors consists of a zinc-binding group (ZBG) that chelates the active site zinc ion, a surface recognition motif (CAP) that interacts with amino acid residues at the entrance of the active pocket, a polar connector unit (CU), and a hydrophobic linker that connect the CAP and ZBG. Structural classes of known HDAC inhibitors include cyclic peptides (romidepsin, apicidin), short-chain fatty acids (butyrate, valproic acid), hydroxamic acids (trichostatin A, vorinostat, panobinostat, belinostat, quisinostat), electrophilic ketones (trifluoromethylketone) and benzamides (entinostat, mocetinostat, tacedinaline/CI-994). Some of these were initially purified from natural sources (romidepsin [106], apicidin [107], trichostatin A [108]) or chemically synthesized (butyrate) without knowing their targets. It was only later discovered that these compounds had HDAC inhibitory activities [105]. Entinostat/MS-275 [109] and vorinostat/SAHA [110] were actively designed using synthetic chemistry and structure-function studies. HDAC inhibitors containing α-amino-ketone groups were discovered using an HDAC fluorescence-based *in vitro* enzyme assay and through a high-throughput luciferase cell-based screen using stably transfected p21-luc H1299 cells [144]. Molecular modeling using X-ray crystal structures and SARs have enabled development of multiple HDAC inhibitors with improved specificity, better bioavailability, higher potency and reduced toxicity.

Over the years, HDAC inhibitors have progressed beyond preclinical studies and have entered clinical

Table 2. Selective small-molecule inhibitors of histone deacetylases, sirtuins and lysine demethylases.

Compound	Target	Discovering strategy	Ref.
HDAC inhibitors			
Romidepsin/depsipeptide	HDACs 1, 2	Purified from natural source†	[105,106]
Apicidin	HDACs 1-3	Purified from natural source†	[105,107]
Trichostatin A	Pan HDACs inhibitor	Purified from natural source†	[105,108]
Butyrate	HDACs 1-5, 7-9	Chemical†	[105]
Entinostat/MS-275	HDACs 1, 2, 3	Synthetic chemistry	[105,109]
Vorinostat/SAHA	Pan HDACs inhibitor	Synthetic chemistry	[105,110]
Trifluoromethylketone	ND	Cell-based HTS	[105,107]
Sirtuin inhibitors			
Splitomicin	SIRT1, 2	Yeast cell-based HTS	[111]
Cambinol	SIRT1, 2	SAR studies on splitomicin	[112]
EX527	SIRT1	Biochemical HTS	[113]
Sirtinol	SIRT1, 2	Yeast cell-based reporter HTS	[114]
Salermide	SIRT1, 2	Molecular modeling on sirtinol	[115]
Tenovin-1	SIRT1	Cell-based reporter HTS	[116]
Tenovin-6	SIRT1	SAR studies on tenovin-1	[116]
AEM1 and AEM2	SIRT2	Biochemical HTS	[117]
SirReals	SIRT2	Biochemical HTS	[118]
KDM inhibitors			
Tranylcypromine	LSD1	Due to LSD1 homology with other MAO	[119]
PG-11144	LSD1	Due to LSD1 homology with PAO	[120,121]
Peptide-based inhibitors	LSD1	Post-assembly modification synthetic strategy	[122]
NCL-1	LSD1	Structure-based design	[123]
Compound 12	LSD1	Structure-based virtual HTS	[124]
Compound 6b	LSD1	Molecular hybridization technique	[125]
ORY-1001	LSD1	Structure-based design and SAR	[126]
NOG and DMOG	2-OG hydroxylases	Due to homology with collagen and HIF PHDs	[127]
Methylstat	KDM4C	Structure-based design	[128]
IOX1	KDM4A	Biochemical HTS and SAR	[129]
PBIT	KDM5A/B	Biochemical HTS	[130]
KDM5-C49, KDM5-C70	KDM5B/C	Biochemical HTS and SAR	[131,132]
TC-E 5002	KDM2A, KDM7A/B	Structure-based design	[133]
BIX-01294, E67, E67-2	KDM7A	Structure-based design	[134]
JIB-04	JmjC KDMs	Cell-based reporter HTS	[135]
GSK-J4	KDM6A/B	Structure-based design and mutation-driven chemoproteomics	[136]
Hybrid/dual KDM inhibitors	LSD1 and JmjC	Structure-based, manual docking	[137]

†Compound was identified without a focused epigenetic screen.
HDAC: Histone deacetylase; HTS: High-throughput screening; JmjC: Jumonji C histone demethylase; KDM: Lysine demethylase; MAO: Monoamine oxidase; ND: Not described; PAO: Polyamine oxidase; PHD: Prolyl hydroxylase; SAR: Structure–activity relationship; SIREAL: Sirtuin-rearranging ligand; SIRT: Sirtuin.

trials. First generation inhibitors such as romidepsin (FK228) and vorinostat (SAHA) have already been approved for clinical use as single agents as well as in combination with standard chemotherapies [145]. Newer HDAC inhibitors with improved substrate selectivity such as entinostat (MS-275) and mocetino-

stat (MGCD0103) are also being evaluated in the clinic alone or in combination with other drugs including the DNMT inhibitor azacytidine [146,147].

Inhibition of sirtuins/class III HDACs

The silent information regulator 2 (Sir2) proteins, sirtuins, are an NAD^+-dependent family of enzymes among which sirtuins 1, 2, 3, 5 and 7 catalyze a deacetylation reaction while SIRT4 and SIRT6 act mainly via ADP-ribosylation [148]. SIRT1 causes epigenetic silencing via deacetylation of histone H1K26, H3K9 and H4K16. In addition to histone substrates, sirtuins also target non-histone proteins such as transcription factors and DNA repair proteins, thereby regulating various biological processes. The role of sirtuins in cancer is complex as these enzymes can be tumor promoting or tumor suppressive depending on the context. SIRT1 and SIRT2 are known to be overexpressed in certain cancer types [149,150] and have also been implicated in multidrug resistance [151]. Consequently, there has been interest in developing specific SIRT1 and SIRT2 inhibitors.

A small-molecule sirtuin inhibitor called splitomicin was identified through a yeast cell-based screen for inhibitors of telomeric silencing [111]. However, due to the instability of this compound limiting its use, the derivative cambinol was subsequently evaluated. This compound, containing a β-naphthol pharmacophore, was shown to have antitumor activity *in vitro* and in Burkitt lymphoma xenografts [112]. An indole compound, EX527, was identified through a high-throughput screen against recombinant human SIRT1 using an assay that measured enzymatic activity on a fluorogenic SIRT substrate [113]. The Schreiber group reported identification of the sirtuin inhibitor sirtinol, using a yeast cell-based high-throughput screen on 1600 unbiased compounds. Since Sir2 proteins are known to cause transcriptional silencing at the telomeric loci, the primary screen was based on yeast Sir2p-mediated silencing of a *URA3* reporter gene integrated into a telomeric locus [114]. Addition of a Sir2p inhibitor caused expression of *URA3* gene and cell death in the presence of FUra. Compounds containing 2-hydroxy-1-naphthaldehyde moiety such as sirtinol were thus identified as a novel class of sirtuin inhibitors. Later, Lara *et al.* performed molecular modeling on sirtinol to develop a stronger inhibitor with better anticancer activity [115]. The new molecule salermide was thus developed by changing the amide moiety in sirtinol to a reverse amide. Salermide was cancer-selective as it did not cause apoptosis in non-tumorigenic fibroblasts. All three SIRT inhibitors described above, namely EX527, sirtinol and salermide have also been tested subsequently by other groups for their SIRT1/2 specificity and anticancer potential [152]. In MCF7 breast cancer cells, SIRT1/2 inhibitors sirtinol and salermide caused acetylation of p53, a target of SIRT1/2, and of tubulin, a SIRT2 target. Both these small-molecule inhibitors resulted in p53-dependent apoptosis. On the other hand, EX527 that is a SIRT1 specific inhibitor failed to cause p53 or tubulin acetylation and MCF7 cancer cell death emphasizing the importance of combined SIRT1/2 targeting.

Using p53 activation as a sensor for compound activity, Lain *et al.* screened 30,000 small molecules in a primary assay that employed T22-RGC-ΔFos-lacZ murine cells expressing β-galactosidase under the control of a p53-dependent promoter [116]. The compound hits obtained from this p53-dependent reporter activation assay were then tested on H1299 cells that lack p53 to filter out those that also caused p53-independent reporter induction. Subsequent hits were prioritized based on their differential toxicity on ARN8 human melanoma cells expressing wild-type p53 compared with normal human dermal fibroblasts (NHDFs). Finally, after testing effects on cell cycle profiles of SKNSH-pCMV cells (expressing functional p53) and SKNSH-DNp53 cells (p53 function abolished by overexpression of a dominant negative form of p53), tenovin-1 was identified. Upon further evaluation, tenovin-1 was found to elevate the amount of p53 protein and p53-downstream target p21CIP/WAF1. This small molecule caused cell death in p53 wild-type BL2 Burkitt's lymphoma cells and ARN8 melanoma cells in culture and also impaired the growth of BL2 or ARN8-derived tumor xenografts. However, tenovin-1's poor water solubility limited its use *in vivo*. Hence, SAR studies were used to guide the synthesis of a water-soluble derivative of tenovin-1 called tenovin-6. In addition to its water solubility, this new molecule showed more potent antitumor activity in ARN8-derived xenograft tumors. In a follow-up study by Li *et al.* on chronic myelogenous leukemia (CML) [153], tenovin-6 increased p53 acetylation and induced apoptosis in leukemia stem cells (LSC) which overexpress SIRT1. Further, tenovin-6 also impaired CML LSC engraftment in immunodeficient mice and inhibited CML progenitor cell growth when combined with the BCR-ABL tyrosine kinase inhibitor imatinib, in an inducible BCR-ABL transgenic mouse model of CML [153].

Recently, two structurally related SIRT2 selective inhibitors were identified through an *in vitro* deacetylation assay-based screening that used full-length SIRT1 and MAL (acetyllysine residue connected to the fluorophore 7-amino-4-methyl-coumarin) as the substrate. These newly identified AEM1 and AEM2 compounds caused apoptosis in p53-proficient non-

small-cell lung cancers and also sensitized them to DNA damaging agents such as etoposide [117]. Following this, in another recent *in vitro* high-throughput screen, fluorophore-labeled acetyl-lysine peptide substrate for human Sirt1–3 was used to identify a family of aminothiazole compounds called Sirtuin-rearranging ligands (SirReals), with SirReal2 having the greatest inhibitory properties [118]. Sirt2-selective binding by SirReal2 was validated using nonlabeled peptidic substrates in a high-performance liquid chromatography (HPLC)-based conversion assay and thermal stability assays. Using crystal structures of Sirt2 in complex with SirReal2, it was determined that SirReal2 binds to the active site of Sirt2 in the extended C-site (EC-site), resulting in structural rearrangement of the active site unveiling a new adjacent binding pocket. SirReal2 significantly increased α-tubulin acetylation in HeLa cells, however further studies will be required to evaluate the antitumor efficacy of these newly identified SirReals *in vivo* [118].

Inhibition of histone demethylases

Like acetylation, methylation marks on histones are now known to be dynamic and can be reversed by the action of histone demethylases. Due to their vital regulatory function, histone demethylases are beyond doubt important mediators in oncogenesis and have been found to be overexpressed, amplified, fused or mutated in many cancer types [154]. Histone demethylases can be classified into the lysine-specific demethylase (LSD) family and the JmjC domain-containing lysine demethylase family.

LSD family inhibitors

The LSD family consists of two members, LSD1 (KDM1A/AOF2) and LSD2 (KDM1B/AOF1). These proteins are flavin adenine dinucleotide (FAD)-dependent monooxidases that catalyze the demethylation of only mono- and dimethylated, but not trimethylated lysines. LSD1 has been implicated in tumor proliferation and metastasis [155], cancer cell metabolism [156] as well as cancer stemness and drug resistance [157]. LSD1 is thus a potential target for cancer therapy.

Due to the homology of LSD1 catalytic domain with other monoamine oxidases (MAO), general inhibitors of MAOs such as pargyline and tranylcypromine (TCP) have been tested for their LSD1 inhibitory potential. An analog of TCP, trans-N-((2-methoxypyridin-3-yl) methyl)-2-phenylcyclopropan-1-amine), was developed to improve potency and selectivity. This analog showed antitumor activity in a MLL-AF9 leukemia mouse model as well as inhibited clonogenicity of primary MLL-AF9 AML patient tumor cultures [119].

Apart from MAOs, LSD1 also shares homology with polyamine oxidases, such as spermine oxidase (SMO/PAOh1). Hence, Huang *et al.* tested known polyamine oxidase inhibitors for their activity on LSD1 inhibition. These unique biguanide and bisguanidine polyamine analogs inhibited LSD1 in human colon carcinoma cells and resulted in re-expression of several aberrantly silenced genes that corresponded with an increase in H3K4me2 activating marks [120]. PG-11144 that belongs to the novel class of long-chain polyamine analogs known as oligoamines, was evaluated for its LSD1 inhibitory activity and anticancer potential [121]. PG-11144 inhibited LSD1 demethylase activity, increased global H3K4me1 and H3K4me2 levels and induced apoptotic cell death in colorectal cancer cells. PG-11144 was also synergistic with the DNMT inhibitor 5-Aza in inhibiting colorectal tumor growth *in vivo*.

Peptide-based LSD1 inhibitors were designed using lysine derivatives based on structural knowledge and known strategies for blocking amine oxidases [122]. A post-assembly modification synthetic strategy was used to increase compound stability. One of these inhibitors containing a propargylamine functionality showed time-dependent inactivation of LSD1 in a H_2O_2 detection biochemical assay. This compound suffered from poor bioavailability but, nevertheless, served as a prototype for the development of other small-molecule LSD1 inhibitors. These new compounds were designed based on X-ray crystal structure data of FAD-trans-2-phenylcyclopropylamine (PCPA) adduct and FAD-N-propargyl lysine peptide adduct in the active site of LSD1. Two compounds in this study dose-dependently elevated H3K4me2 levels in HEK293 cells and suppressed cell growth of human cancer cell lines [123]. One of these compounds later called NCL-1, reduced proliferation of stem-like glioma cells and also caused inhibition of glioma xenograft tumor growth [158]. In a recent study, NCL1 also increased H3K9me2 at the promoters of androgen-responsive genes, suppressed proliferation of prostate cancer cells *in vitro* and decreased tumor growth and vascularity in xenografts [159].

Another class of LSD1 inhibitors comprising the N′-(1-phenylethylidene)-benzohydrazide series was identified using a high-throughput structure-based virtual screen of a compound library containing approximately 2 million small molecules [124]. Lead compound 12 is a noncompetitive LSD1 inhibitor that showed anticancer activity against endometrial, breast, colorectal and pancreatic cancer cell lines. Compound 12 caused global increases in H3K9me2 in an androgen-sensitive prostate cancer cell line, confirming that the antiproliferative effects of compound 12 were on target [124].

A different strategy to design more selective LSD1 inhibitors with minimal side effects involves the molecular hybridization technique. Using this approach, two or more drug pharmacophores can be combined into a single hybrid molecule with improved properties. In a study published this year, a series of small-molecule LSD1 inhibitors were designed using the previously established aminothiourea and propargyl pharmacophores and linking them through a pyrimidine moiety, to obtain synergistic activity. SAR studies were performed to assess the importance of different subgroups in LSD1 inhibitory activity. The aminothiourea subgroup was important because of the steric hindrance around the pyrimidine ring whereas propargyl group was essential owing to the N5 of the flavin causing nucleophilic attack to the propargyl scaffold. Orally active pyrimidine–thiourea-based compound 6b was then evaluated for its anticancer activity. This compound dose dependently elevated H3K4me1/me2 and H3K9me2 levels, induced apoptosis in LSD1 overexpressing gastric cancer cells and also delayed gastric tumor growth *in vivo*. Furthermore, it also inhibited cell migration and reduced the number of metastatic nodules in a melanoma cell line derived lung metastatic model [125].

LSD1 inhibitors are thus attractive therapeutic targets for a variety of cancers and in the last 2 years, one of them has been approved for Phase I/IIA clinical trials. The orally bioavailable clinical compound ORY-1001 was developed by Oryzon Genomics using computational models based on reported X-ray structures and SAR. Treatment of MLL-AF9 transformed mixed lineage leukemia cells with ORY-1001 resulted in time/dose dependent H3K4me2 accumulation at LSD1/KDM1A target genes and suppressed cancer cell growth both *in vitro* and *in vivo* [126]. ORY-1001 is now under evaluation in patients with relapsed or refractory acute leukemia (EudraCT Number: 2013–002447–29).

JmjC domain-containing lysine demethylase family inhibitors

JmjC domain-containing proteins constitute the largest family of histone demethylases with about 30 known human proteins, 17 of which have defined enzyme activities. Enzymatic reaction involves an oxidative mechanism that is dependent on α-ketoglutarate (2OG) and iron, to hydroxylate methyl lysine, releasing formaldehyde thus causing demethylation. Contrary to LSD enzymes, JmjC domain containing enzymes can demethylate trimethylated lysine in addition to the di- and mono-methyl groups.

Several members of the JmjC histone demethylase family are known to be amplified or overexpressed in human cancers. This includes enzymes that demethylate the repressive H3K9 and H3K27 methylation marks as well as family members that remove the H3K4 and H3K36 activating marks, thereby resulting in transcriptional activation of oncogenes or repression of tumor suppressors, respectively. For instance, the H3K9 demethylases KDM3A and KDM3B were found to be elevated in human bladder carcinomas and acute lymphoblastic leukemia, respectively [160,161]. This leads to transcriptional activation of KDM3A-induced leukemogenic oncogene *lmo2* or KDM3B induced *HOXA1* expression. Similarly, the H3K4 demethylase KDM5B/JARID1B/PLU-1 was shown to be upregulated and involved in regulating transcriptional activity of androgen receptor (AR) in prostate cancer and tumor suppressor gene *BRCA1* in breast cancer [162,163]. Demethylases belonging to the KDM4/JMJD2 family have been found to be amplified or overexpressed among others in breast cancer, squamous cell carcinoma and medulloblastoma [142,154]. Furthermore, histone demethylases are also implicated in cancer stem cells and drug resistance. For instance, KDM5A/JARID1A is involved in reversible drug-tolerant cancer cells surviving EGFR TKIs and KDM5B/JARID1B in multidrug resistant melanoma cells [164,165]. Recently, KDM2B/NDY1 demethylase was shown to be associated with breast cancer stemness and high recurrence rate after therapy [166]. Jumonji histone demethylases are thus important targets for therapeutic intervention.

Among the first inhibitors tested for JmjC demethylases were the broad-spectrum 2OG oxygenase inhibitors that were known to inhibit collagen and HIF prolyl hydroxylases (PHD). This includes the N-oxalyl amino acid-based inhibitors, namely *N*-oxalylglycine (NOG) and its cell-permeable derivative dimethyl N-oxalylglycine (DMOG) that act by hindering oxygen binding to the active site iron thereby blocking catalysis [127]. Similarly, pyridine dicarboxylates (2,4-PDCA) and 2,2′-bipyridine derivatives have also been used for Jumonji enzyme inhibition *in vitro*. These compounds are helpful research reagents yet do not have good therapeutic potential due to their lack of cell permeability and/or specificity to JmjC demethylases [127].

In order to improve selectivity, compounds that combined the α-ketoglutarate mimic (co-factor) and a methyllysine mimic (substrate) were designed. The methyl ester prodrug form of the designed compound, called methylstat, inhibited growth of KDM4C/JMJD2C-sensitive esophageal carcinoma cell line while globally increasing H3K4me3 and H3K9me3 levels [128].

Another class of JmjC demethylase inhibitors contains the 8-hydroxyquinoline group. This series of compounds was identified using quantitative high-throughput screening (qHTS) of >200,000 compounds by employing a real-time fluorescence-based assay that detects formaldehyde formation from the demethyl-

ation reaction. These 8-HQ compounds act by binding to the active site Fe(II). The derivative selected based on SAR studies, 5-Carboxy-8-HQ (IOX1), inhibited KDM4A/JMJD2A in HeLa cells [129]. An n-octyl ester derivative of IOX1 was later developed to improve cell permeability [167]. This serves as a cell-permeable tool compound for further investigational studies.

To identify small-molecule inhibitors that selectively target KDM5/JARID1 family of histone demethylases, an AlphaScreen platform-based high-throughput screen was performed on approximately 15,000 compound library. The assay detected demethylation of a biotinylated H3K4me3 peptide by JARID1B. The screen yielded PBIT, which specifically inhibited JARID1A/B, increased H3K4me3 levels and suppressed proliferation of breast cancer cells [130]. The pharmaceutical company EpiTherapeutics has also developed potent JARID1 inhibitors that increase cellular H3K4me3 levels and possess anticancer activity [131–132,154]. The small-molecule KDM5-C49 was determined to have potent KDM5C/JARID1C inhibitory activity in histone lysine demethylase AlphaLISA assays. However, cellular permeability of this compound is limited due to the highly polar carboxylate group. Hence to mask the polarity of this acid group, a methyl ester derivative of KDM5-C49 was developed as a prodrug and called KDM5-C70. This cell permeable molecule inhibited KDM5B activity in *in vitro* enzyme assays, inhibited demethylation of H3K4 in U2OS human osteosarcoma cell line, and also suppressed proliferation of MCF7 breast cancer cells.

Recently, a new hydroxamate compound, 6j, and its prodrug 7j, have been identified as selective KDM5A/JARID1A inhibitors [133]. Using knowledge obtained from previous docking studies of hydroxamate-based JMJD2 and JHDM1 inhibitors and structural insights into the unique features of the JARID1A catalytic pocket, Itoh *et al.* screened a library of hydroxamate analogs using a H3K4me3 peptide substrate and a formaldehyde dehydrogenase-coupled assay, to identify JARID1A inhibitors. After performing binding simulations and structural optimizations, compound 6j was derived. Its methyl ester prodrug 7j was synthesized to improve cellular permeability. This compound caused a dose-dependent increase in global H3K4me3 in A549 lung cancer cells, without affecting H3K9me3 and H3K27me2 levels. 7j did not affect the proliferation of A549 cells but it led to synergistic cell growth inhibition when combined with the HDAC inhibitor, vorinostat.

Other important candidate anticancer agents are inhibitors of the KDM2/7-subfamily. Daminozide, which was used as an agrochemical due to its plant growth retardant properties, was found to be active against the KDM2/7 subfamily via active site metal chelation [168]. However, its 1,1-dimethylhydrazine structure was determined to be genotoxic. Hence, to identify new compounds which lack this genotoxic structure, a series of hydroxamate derivatives were synthesized by studying the crystal structure of KDM7B. Compound 9 later called TC-E 5002, inhibited KDM2A and KDM7A/B, caused global H3K27me2 accumulation, decreased E2F1 mRNA expression and resulted in a G_0/G_1 phase cell cycle arrest in HeLa and KYSE150 cells [169].

The Cheng group recently demonstrated that BIX-01294 (a diazepin-quinazoline-amine derivative) and its E67 and E67–2 analogs are also inhibitors of KDM7A/KIAA1718 [134]. These small molecules were originally designed as potent inhibitors of H3K9 methyltransferases G9a and GLP [27]. Since both lysine methyltransferases and demethylases recognize lysines in methylated and unmethylated states, Upadhyay *et al.* explored whether BIX-01294 and its derivatives could also function as H3K9 demethylase inhibitors [134]. These small molecules were indeed found to inhibit KDM7A activity without affecting KDM5C/JARID1C demethylase activity, confirming specificity for H3K9 demethylase inhibition. Guided by KIAA1718-E67 co-crystal structure complex, the BIX-01294 analog E67–2 was developed by deleting the benzylated six-membered piperidine ring moiety in E67. The resulting derivative showed higher differential selectivity against KDM7A demethylase over the GLP methyltransferase. Further studies would be required to evaluate the anticancer activity of BIX-01294 and its derivatives in KDM7A overexpressing cancers [134].

Studies conducted in our laboratory recently identified a novel, cell permeable inhibitor of JmjC demethylases, JIB-04, consisting of a pyridine hydrazone structure. We identified this small molecule in a cell-based screen on NCI's diversity set of approximately 3000 compounds, using a GFP reporter-based locus derepression (LDR) assay [135]. JIB-04 inhibited activity of several JmjC demethylases in purified *in vitro* systems without affecting other epigenetic/hydroxylase enzymes including LSD1, PHD and TET1. Importantly, JIB-04 induced transcriptional changes selectively in cancer cells and suppressed lung cancer cell growth *in vitro* and *in vivo*, while also prolonging the survival of mammary tumor-bearing mice. Tumors treated with JIB-04 had reduced H3K9me3 demethylase activity. Thus, JIB-04 was the first Jumonji inhibitor to show *in vivo* efficacy [135]. Subsequently, using genetically encoded H3-K9 histone methylation biosensors, Sekar *et al.* showed that JIB-04 caused a dose-dependent global increase in H3K9 methylation levels in treated HEK293T cells [170]. In a further study, Van Rechem *et al.* demonstrated the role of KDM4/5 in protein synthesis and showed that JIB-04 caused defects in translation initiation and sensitized cancer cells to mTOR inhibitors [171].

The small-molecule JmjC inhibitor GSK-J1 was derived from the GlaxoSmithKline collection of approximately 2 million small molecules using a structure-function approach employing the crystal structures of KDM6B/JMJD3 [136]. Key residues of the H3K27me3 peptide–enzyme complex as determined from the crystal structure were mutated to identify the residues important for substrate recognition. This mutation-driven chemoproteomics approach also enabled further optimization of weakly active compounds. To improve cellular permeability, an ethyl ester derivative of GSK-J1 was synthesized and called GSK-J4. This small-molecule inhibitor was first studied in the context of proinflammatory macrophage response by Kruidenier *et al.* and was later used in anticancer studies by other groups [136,172–174]. In JMJD3 overexpressing human T-ALL cells, GSK-J4 caused transcriptional changes that overlapped those detected from JMJD3 shRNA knockdown, increased H3K27me3 levels at repressed genes, led to cell cycle arrest and increased apoptosis [174]. In a different study by Hashizume *et al.*, GSK-J4 increased H3K27me2 and me3 levels and inhibited cell growth in K27M-mutant brainstem glioma cell lines [173]. *In vivo* studies confirmed that GSK-J4 selectively inhibited growth of K27M mutation-bearing pediatric brainstem glioma xenografts, led to significantly higher tumor cell H3K27me3 positivity and increased survival of treated mice [173]. Grasso *et al.* recently reported that GSK-J4 also synergized with the HDAC inhibitor panobinostat in H3K27M diffuse intrinsic pontine glioma (DIPG) cells [172].

Finally, there has also been interest in generating pan histone lysine demethylase inhibitors targeting both LSD1 and the JmjC family, because of the co-expression of these enzymes in some cancers, including prostate cancer [137]. After studying crystallographic structures of both the LSDs and JmjC KDMs and using a manual docking strategy, hybrid LSD1/JmjC inhibitors were synthesized [137]. Hybrids were derived by linking the structure of LSD1 inhibitor tranylcypromine with 2,2′-bipyridine based or 8-hydroxyquinoline derived JmjC inhibitor templates. Hybrid compounds 2 and 3 increased both H3K4me2/3 and H3K9me3 methylation and induced apoptosis selectively in prostate and colon cancer cells, without affecting non-cancerous cells. Such dual KDM inhibitors could thus be potentially useful in particular cancer types [137]. All these studies involving the development and use of LSD1 and JmjC inhibitors thus emphasize that histone demethylases are targetable and have promising potential for anticancer therapy as single agents or in combination.

Future perspective

With several inhibitors of histone writers and erasers currently in early phase clinical trials for targeted subpopulations, it is likely that good patient response for specific indications will be seen. Beyond targeted application to subsets of patients whose tumors have defined epigenetic susceptibilities, the inhibitors described here may also prove to be effective foundations for combination therapies. In particular, the role of epigenetic enzymes in the development of resistance to both chemotherapies and radiation is emerging and may define a new vulnerability across several types of cancer. Although not imminent, it is not hard to envision future combinations that will both target the primary tumor and the development of therapeutic resistance by including epigenetic modulators in combination cocktails with standard therapies.

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Executive summary

- Histone writers (including HAT, KMT and PRMT enzymes) and histone erasers (including HDAC, Sirtuin and KDM enzymes) are deregulated in many tumor types and constitute good drug targets.
- Inhibitors of histone acetyl transferases (HATs), lysine methyltransferases (KMTs), histone deacetylases (HDACs) and lysine demethylases (KDMs) have been developed to block the aberrant action or hyperactivity of these epigenetic enzymes in cancer, using *in silico*, biochemical, structural and cell-based approaches.
- Several inhibitors have advanced from the discovery stage to preclinical evaluation including mouse cancer models, and have shown antitumor efficacy and survival benefits.
- A few small molecules targeting histone methylation pathways are in early clinical trials and, if successful, will represent the next advance in epigenetic therapy beyond the already approved DNA methyltransferase and HDAC inhibitors.

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Review
Special Focus Issue: Chemical Biology

Future Medicinal Chemistry

Recent developments and applications of clickable photoprobes in medicinal chemistry and chemical biology

Photoaffinity labeling is a well-known biochemical technique that has grown significantly since the turn of the century, principally due to its combination with bioorthogonal/click chemistry reactions. This review highlights new developments and applications of clickable photoprobes in medicinal chemistry and chemical biology. In particular, recent examples of clickable photoprobes for target identification, activity- or affinity-based protein profiling (ABPP or AfBPP), characterization of sterol– or lipid–protein interactions and characterization of ligand-binding sites are presented.

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Central to the biochemical method of photoaffinity labeling (Figure 1) is the development and utilization of compounds called photoaffinity ligands (**1**), also known as photoprobes [1]. The general composition of a photoprobe includes a ligand of interest, as a specificity/affinity unit, that is typically derivatized with a reporter tag (Tag) and a photoreactive group (PRG). In most applications of photoaffinity labeling, cell lysates or cells are first treated with the photoprobe, and then time is allowed for the photoprobe to bind reversibly with its targets. In this regard, the ligand of interest is responsible for forming specific, high-affinity reversible complexes (**3**) upon probe binding to target biological macromolecules (**2**) within the sample. The sample is then irradiated with UV light of a specific wavelength to convert the photoreactive group into a highly reactive intermediate. This intermediate will then ideally react rapidly and irreversibly (i.e., covalently) with a nearby amino acid within the target protein (i.e., entity X in **3** and **4**).

It is important to note that photoaffinity labeling can result in nonspecific labeling of proteins, especially if high concentrations of the photoprobe are used. As a result, photolabeling experiments should be conducted both in the presence and absence of the parent compound, or another biologically active analog, as a competitor in order to distinguish specific from nonspecific labeling. After crosslinking of the photoprobe to its targets, if live cells are used as the biological sample, the cells are then lysed, and the experimental protocol proceeds toward separating tagged, probe-labeled proteins from the rest of the biological sample via the reporter tag. For example, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) readily allows visualization and analysis of probe-labeled proteins using reagents, techniques and equipment specific for the reporter tag. Probe-labeled proteins can then be cut from the gel and subjected to enzymatic digestion in order to produce peptide fragments (**5**). In turn, these fragments can be analyzed by MS to aid in the identity of probe-labeled proteins. Furthermore, if sufficient amounts of purified target protein is available, in some cases it is possible to determine the amino acid sequence of the probe-labeled peptide fragments. This then allows one to work backward to gain structural information regarding binding sites within the target protein. As a result of this experimental workflow, photoaffinity labeling has a number of powerful applications, whether it be at the macrolevel (i.e., **4**; e.g., target identification of hit compounds originating from screening campaigns, affinity-based protein profiling (AfBPP), imaging applications) or

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Key terms

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Biochemical technique commonly used to separate biological macromolecules, such as nucleic acids and proteins, based on their electrophoretic mobility. The electrophoretic mobility of a biological macromolecule is generally a function of the conformation, charge and size of the compound. In particular, SDS is an anionic detergent that linearizes proteins and generally imparts an even distribution of negative charge per unit mass of protein. This then facilitates the separation of proteins by approximate size during electrophoresis.

Click chemistry: Analogous to nature, which synthesizes compounds by efficiently joining small modular units together, click chemistry refers to a group of chemical reactions that meet a significant number of the following criteria: modularity, wide scope, high yielding, stereospecific, green, physiologically stable and high atom economy with a large thermodynamic driving force. Additionally, click chemistry reactions are generally simple to perform, feature readily available reagents and starting materials, and typically involve simple product isolation via nonchromatographic methods. While there are a number of chemical reactions that meet most of these criteria, the copper-catalyzed Huisgen 1,3-dipolar cycloaddition of terminal alkynes with azides represents the most common click chemistry reaction.

Phenotypic screening: Type of screening typically used in drug discovery and biological research to identify compounds that alter the phenotype (i.e., characteristics or traits) of an organism or cell in a desired manner. Simple phenotypic screens traditionally involve cell lines where a single parameter is typically monitored (e.g., production of a particular protein, cellular death, etc.). However, phenotypic screening can also feature animal-based systems or model organisms (e.g., mouse, zebrafish, fruit fly) to evaluate the effects of a compound in a fully intact biological system.

microlevel of analysis (i.e., **5**; e.g., binding site location and mapping, and elucidation of ligand–target interactions) [2].

The most common photoreactive groups employed in the design of photoprobes are benzophenones (**6**), aryl azides (**7**), and aromatic (**8**) and aliphatic diazirines (**9**) (Figure 2A). Upon photoactivation, these functional groups form highly reactive triplet ketyl diradicals, nitrenes, and carbenes, respectively, which facilitate irreversible covalent attachment of the photoprobe to the target (i.e., the conversion of **3** to **4**). For a discussion of the advantages, disadvantages, and chemistry associated with these photoreactive groups, the reader is advised to consult the most recent review on photoaffinity labeling [1]. Additionally, reporter tags within designed photoprobes help facilitate isolation, measurement, detection, and visualization of probe-labeled targets (Figure 2B). In this regard, affinity tags such as biotin (**10**), fluorophores such as TAMRA (**11**), and epitope tags (e.g., **12**, a FLAG peptide) represent long-established options for researchers as reporter tags [3]. However, it should be noted that moieties **10–12** can be unfavorable for direct incorporation into photoprobes. This is because these entities are rather large in size, generally noncell permeable and more likely to cause steric disruption of key interactions between the ligand component (as a specificity/affinity unit) and the biological target [4]. Alternative to moieties **10–12**, radioactive isotopes (e.g., **13**) can be used as small reporter tags for detection purposes. However, these tags have drawbacks as well, including practical concerns of special handling, potentially short half-lives due to relatively fast degradation and lack of an affinity handle for enrichment of probe-labeled targets.

As a result of the previously mentioned disadvantages associated with incorporating large moieties **10–12** directly into the design of photoprobes, an alternative experimental strategy has since appeared (Figure 3A). This strategy features 'clickable photoprobes' (**16**), which contain a bioorthogonal/click chemistry functional group, typically an aliphatic azide (**14**) or a terminal alkyne (**15**), as an 'indirect' tag within the photoprobe. The strategy, also known as tandem photoaffinity labeling-bioorthogonal conjugation [5], relies on photoaffinity labeling of target proteins followed by the employment of a bioorthogonal conjugation reaction to affix a tag of choice specifically to the click chemistry handle within the photoprobe. The main bioorthogonal conjugation reactions that have proved successful in this experimental strategy are the copper-catalyzed Huisgen 1,3-dipolar cycloaddition [6] and the Staudinger-Bertozzi ligation [7] (Figure 3B). However, recent examples involving oxime click chemistry and copper-free tetrazine ligation with strained alkenes have started to emerge (*vide infra*). Subsequently, this two-step experimental strategy has proved highly advantageous in a number of settings, principally because the bioorthogonal/click chemistry functional group within the photoprobe is typically small and easily installable. As a result, this 'clickable' functional group is less likely to negatively affect cell permeability or biological activity of the photoprobe relative to the parent compound, namely by not disrupting key ligand–target interactions. Furthermore, tandem photoaffinity labeling-bioorthogonal conjugation allows flexibility with respect to the choice of the tag, thus allowing the researcher to use the same clickable photoprobe for multiple applications (e.g., attachment of a fluorophore for in-gel fluorescence detection, biotin for enrichment/identification, etc.). The only apparent disadvantage associated with this experimental strategy is the yield of the bioorthogonal conjugation reaction step, which can be variable depending on the reaction and biological system employed. In par-

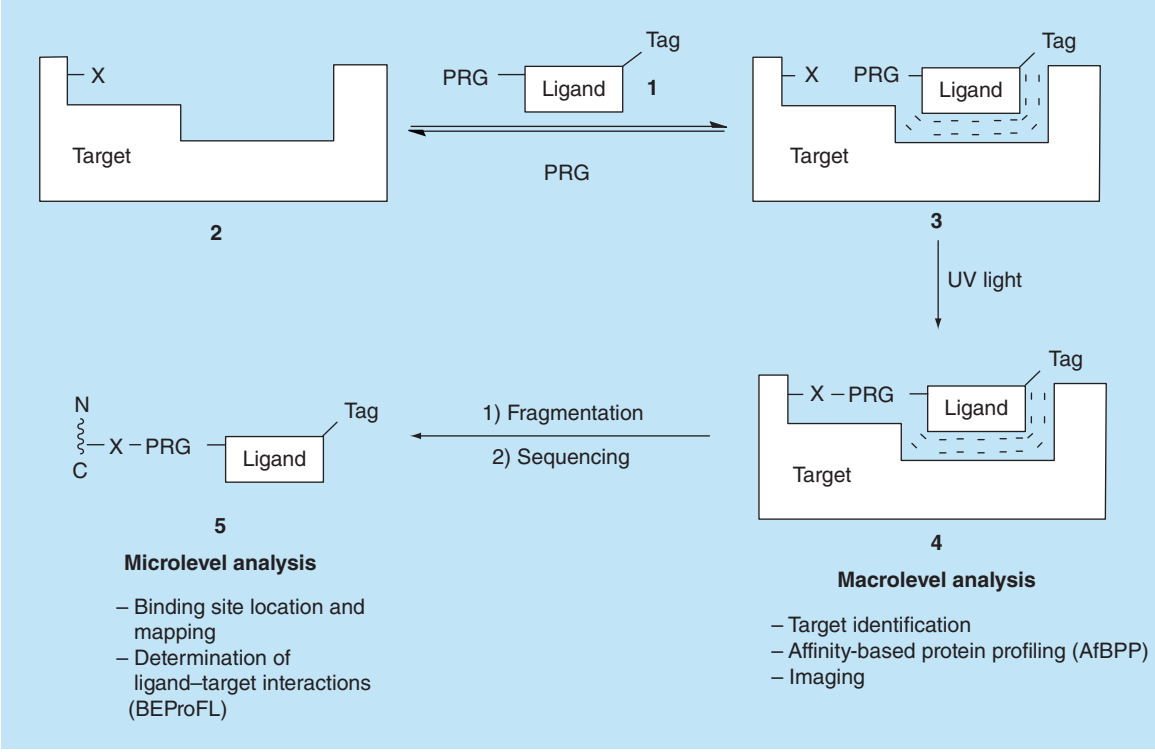

Figure 1. Schematic representation and applications of photoaffinity labeling.
PRG: Photoreactive group.

ticular, higher yields have generally been observed for aliphatic azide chemical reporters/tags upon copper-catalyzed Huisgen 1,3-dipolar cycloaddition versus Staudinger-Bertozzi ligation [8]. However, the numerous advantages associated with this experimental strategy frequently trump the concern over variability in yield of the bioorthogonal conjugation reaction step.

It should be noted that a review of tandem photoaffinity labeling-bioorthogonal conjugation, particularly with respect to medicinal chemistry and chemical biology, has been previously published [5]. Rather than duplicate references from that publication, it is the intent of this review to highlight examples of clickable photoprobes in medicinal chemistry and chemical biology published since that time. In particular, this review will focus on the development and application of clickable photoprobes in the areas of target identification, affinity-based protein profiling (AfBPP), characterization of sterol– or lipid–protein interactions and determination of ligand-binding sites.

Clickable photoprobes for target identification of hit compounds originating from screening campaigns

It is well established that high-throughput screening [9] and phenotypic screening [10,11] play important roles in the discovery of new drugs and chemical probes [12]. In particular, one of the biggest technical challenges in medicinal chemistry and chemical biology is identifying the biologically relevant targets of hits that arise via phenotypic (i.e., organismal or cell) screening. In this regard, clickable photoprobes have facilitated more rapid target identification over the past decade, particularly for hit compounds originating from screening campaigns.

Aryl azide-based probes

A number of clickable photoprobes have been designed and synthesized featuring an aryl azide photoreactive group to facilitate target identification of a hit compound discovered from screening (Figure 4). With respect to the area of infectious disease, piperazine **17** was found to specifically inhibit infection by vesicular stomatitis virus particles pseudotyped with the Ebola virus Zaire glycoprotein [13]. Subsequent hit-to-lead optimization of piperazine **17** eventually led to the development of clickable photoprobe **18**, which features an aryl azide photoreactive group and a unique aryl alkyne click chemistry handle (i.e., most clickable photoprobes feature aliphatic terminal alkynes as chemical reporters, not aryl terminal alkynes, presumably because the latter are more sluggish in bioorthogonal conjugation reactions). In particular, previous functional studies strongly suggested the lysosome membrane protein Niemann-Pick C1 (NPC1) as the target for antiviral compound **17** [13]. This was subsequently confirmed by subjecting photoprobe **18** to tan-

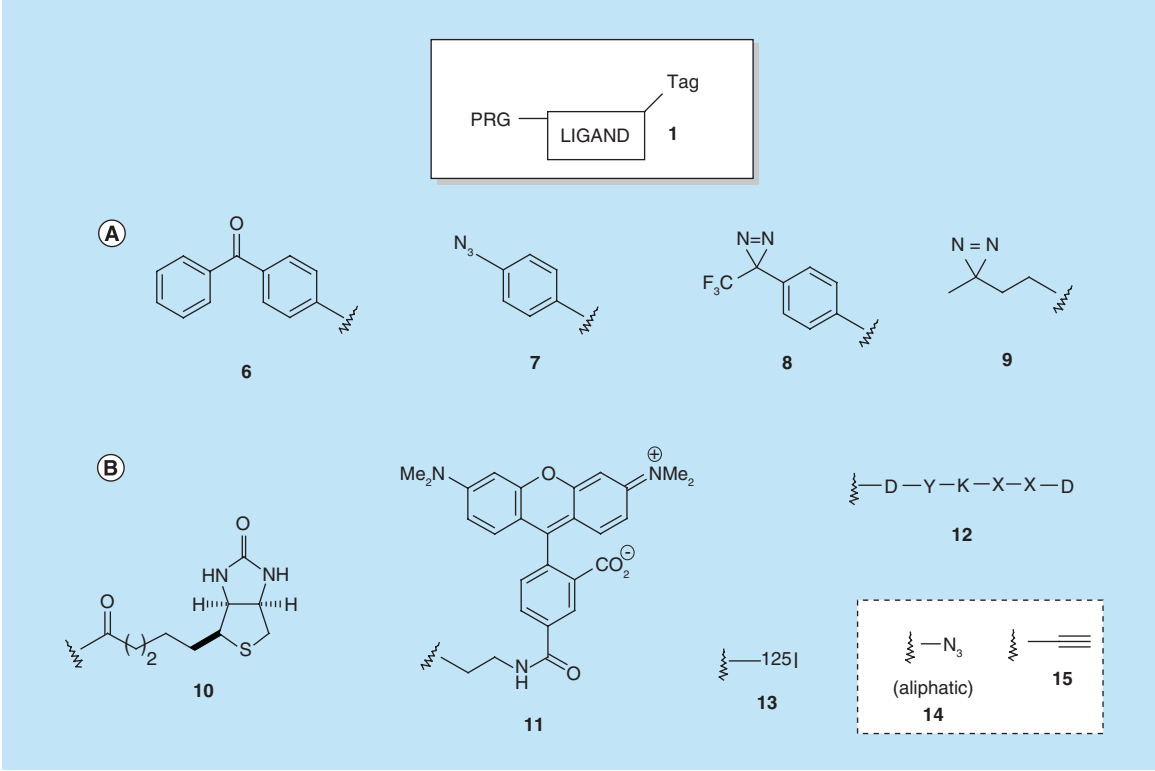

Figure 2. (A) The most common photoreactive groups and (B) reporter tags used in clickable photoprobes.
PRG: Photoreactive group.

dem photoaffinity labeling-bioorthogonal conjugation in late endosomal/lysosomal membranes from Chinese hamster ovary fibroblasts expressing NPC1 [14]. In a similar light, semicarbazone **19** was found to protect cells from anthrax lethal toxin via a high-throughput phenotypic screen [15]. A structure–activity relationship (SAR) study was then performed with the goals of improving the potency of semicarbazone **19** and developing photoaffinity ligands to facilitate identification of proteins involved in anthrax toxin cellular entry [16]. Unfortunately, the SAR of semicarbazone **19** proved to be rather tight and relatively flat, wherein synthesized clickable photoprobe **20** did not offer any protection of cells to anthrax toxin entry at any concentration tested. As a result, this group is currently pursuing azide-based photoaffinity probes bearing radioisotope tags in order to better understand the mechanism of anthrax toxin cellular entry.

Key term

Stable isotope labeling by/with amino acids in cell culture (SILAC): A technique in quantitative proteomics using MS and nonradioactive isotopic labeling to detect differences in protein abundances among samples. In general, two populations of cells are cultivated in cell culture, one of them is fed growth medium containing normal amino acids, while the other is fed growth medium containing amino acids labeled with stable, nonradioactive heavy isotopes. In this way, pairs of chemically identical proteins of different stable isotope composition can be differentiated via their mass difference upon combination of the cell populations. In particular, analysis of the ratio of the peak intensities in the mass spectrum for such pairs directly correlates with the abundance ratio for the two proteins.

In the area of cancer research, there is significant interest in sensitizing compounds that can be used in combination with toxic chemotherapeutic agents to overcome the problem of drug resistance. In this regard, diamide **21** was found to chemosensitize various cancer cell lines to etoposide-induced apoptosis upon screening a commercial compound library [17]. In order to identify the cellular targets of diamide **21**, subsequent derivatization with a photoreactive aryl azide and an alkyne click chemistry handle provided photoprobe **22**. Interestingly, photoprobe **22** displayed significantly higher chemosensitizing potency when pharmacologically compared with diamide **21**, and almost exclusively labeled a single target upon photoaffinity labeling using HeLa or MDA-MB-231 cells. Using a stable isotope labeling by/with amino acids in cell culture (SILAC) approach, this single target was ultimately confirmed to be protein disulfide isomerase (PDI). In particular, diamide **21** represents a rather unique and selective reversible PDI inhibitor (i.e., previously discovered PDI inhibitors were irreversible compounds that lacked selectivity), providing

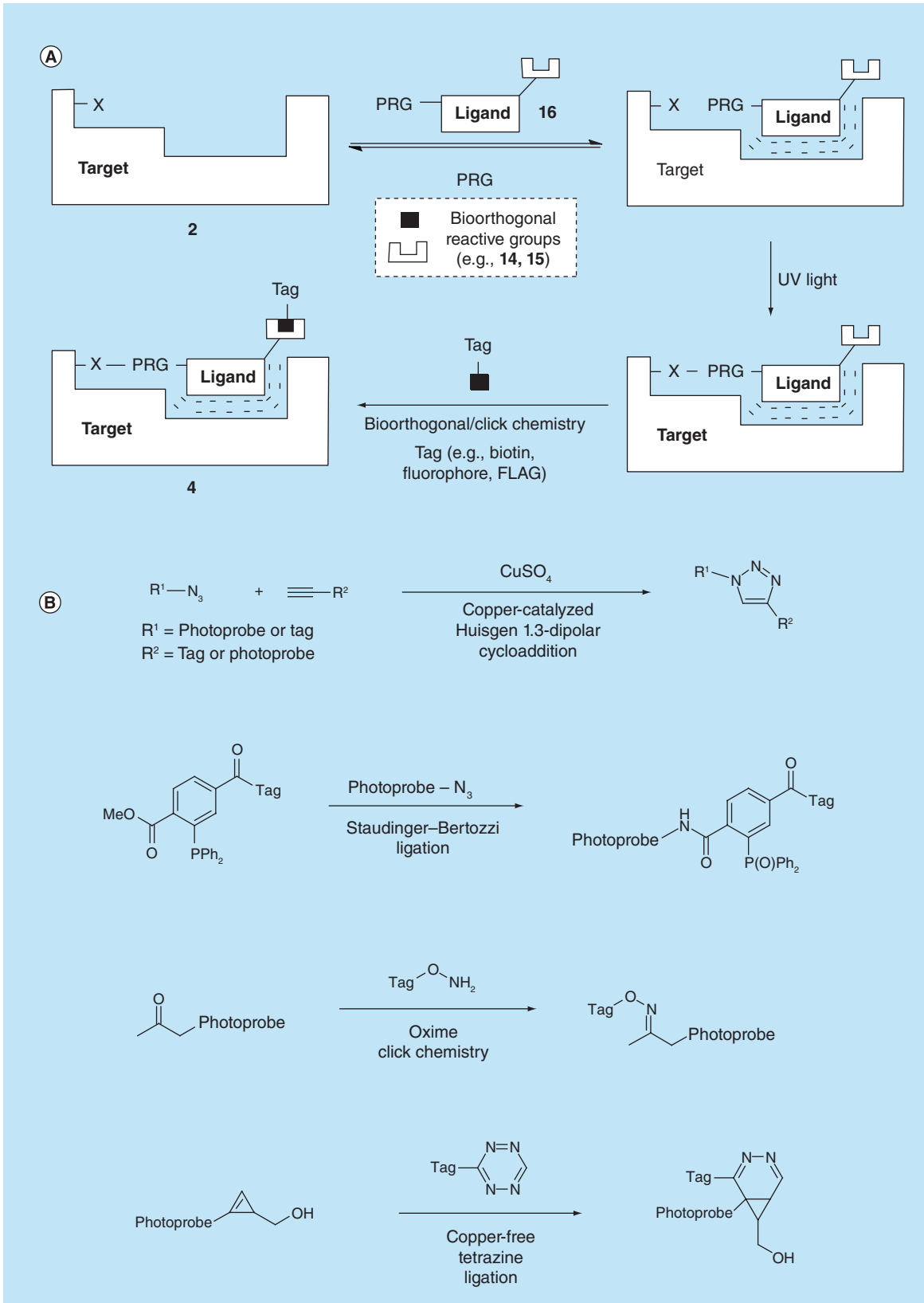

Figure 3. (A) The experimental strategy of tandem photoaffinity labeling-bioorthogonal conjugation and (B) examples of bioorthogonal conjugation reactions successfully used in this strategy.
PRG: Photoreactive group.

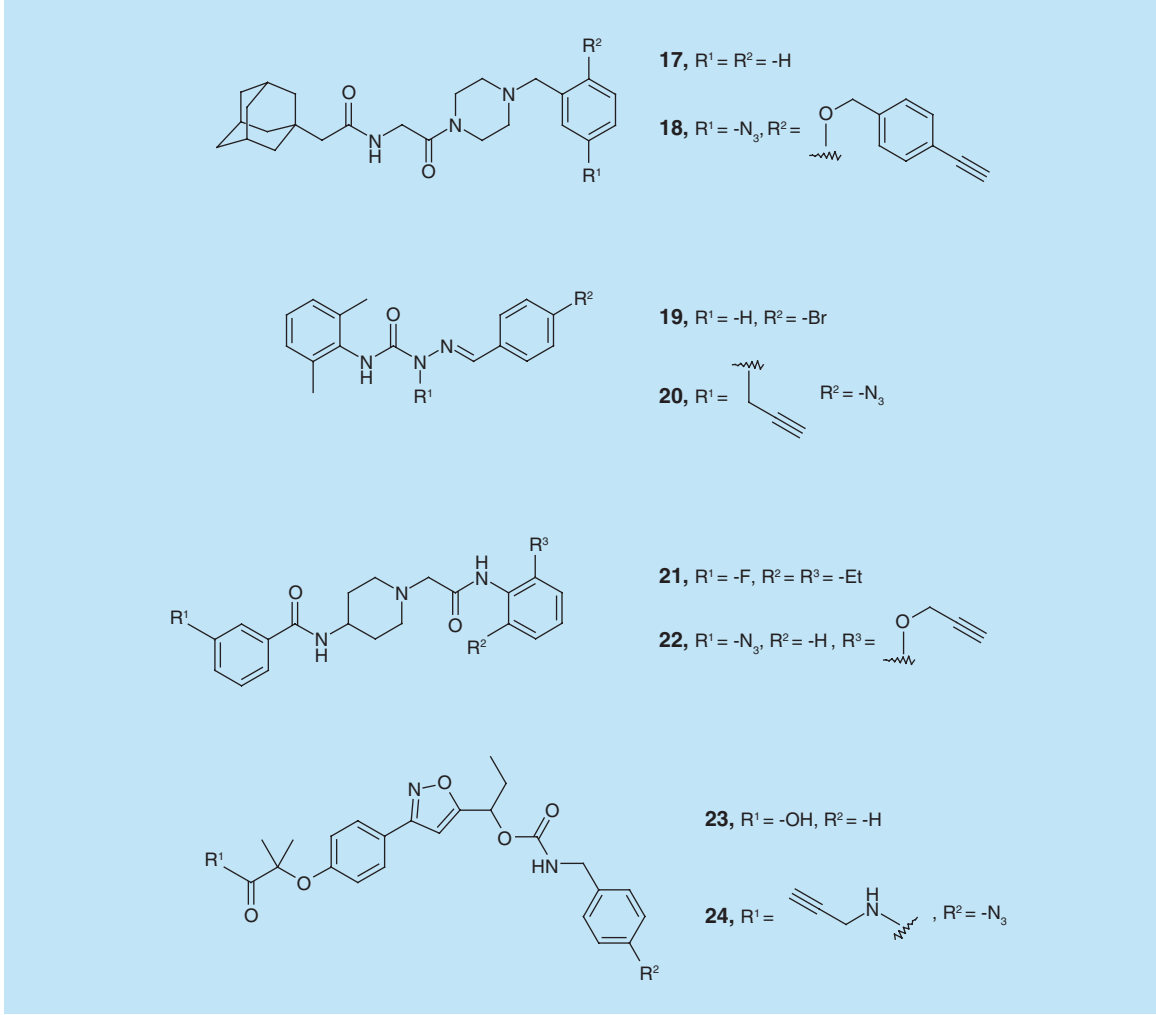

Figure 4. Lead compounds obtained from screening campaigns and their clickable aryl azide-based photoprobe derivatives for target identification.

further impetus for the pursuit of such compounds as potential anticancer agents.

In the area of diabetes, isoxazole **23** was identified as a small-molecule enhancer of cellular glucose uptake upon screening a 3000-member library synthesized by diversity-oriented synthesis. Target identification was then pursued using a fluorescence-guided approach involving clickable azide-based photoprobe **24** [18]. In particular, photoprobe **24** stemmed from SAR studies and retained its bioactivity when compared with isoxazole **23**. Subsequent tandem photoaffinity labeling-bioorthogonal conjugation in differentiated 3T3L1 adipocytes identified PPAR-γ, a well-known protein associated with insulin sensitization, as a potential biological target of these isoxazoles. Indeed, subsequent follow-up using a cell-based PPARγ-luciferase transactivation assay confirmed isoxazoles **23** and **24** as PPAR-γ partial agonists with moderate efficacy. Upon identification of PPAR-γ as a likely target for these compounds, subsequent rational optimization then led to a compound with 4000-fold higher potency in terms of cellular glucose uptake. Additionally, a correlation between the stereochemistry of optimized compounds and bioactivity was elucidated during the course of this work.

Benzophenone-based probes

Alternative to aryl azide based compounds, a number of benzophenone-based clickable photoprobes have been generated with the intention of determining the biological targets of hit compounds originating from screening endeavors (Figure 5). For example, aminopropyl carbazole based compound P7C3 (**25**) originated via an *in vivo* screen aimed at identifying compounds that could enhance hippocampal neurogenesis in adult mice [19]. In turn, numerous derivatives of P7C3 were synthesized, and in general, this compound class fosters the survival of neurons in a variety of rodent models of nerve cell injury or neurodegeneration. Subsequently, clickable benzophenone photoprobe **26** was found to target NAMPT via tandem photoaffinity labeling-

bioorthogonal conjugation [20]. In particular, NAMPT represents the rate-limiting enzyme in the biochemical conversion of nicotinamide to nicotinamide adenine dinucleotide (NAD). Further pharmacological assays subsequently confirmed NAMPT as the likely biological target of numerous P7C3 analogs, wherein it is believed these compounds increase NAD levels via NAMPT-mediated salvage. In short, this work continues to highlight the promise of P7C3 compounds as drug candidates for diseases such as Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease.

In a similar light to P7C3 and adult neurogenesis, KHS101 (**27**, Figure 5) was found to selectively accelerate a neuronal differentiation phenotype. Specifically, KHS101 stemmed from a phenotypic screen and SAR study aimed at identifying compounds that could induce neuronal differentiation of cultured rat hippocampal neural progenitor cells (NPCs) [21]. In order to elucidate the specific target of KHS101 in NPC lysates, tandem photoaffinity labeling-bioorthogonal conjugation was pursued using benzophenone-alkyne **28** [22]. Subsequent 2D SDS-PAGE followed by MS led to the identification of transforming acidic coiled-coil-containing protein 3 (TACC3) as a probable biological target for KHS101. This target was subsequently confirmed by western blotting using an antibody specific for TACC3, as well as experiments involving purified recombinant rat TACC3 protein. This work suggests that appropriate modulation of TACC3 can accelerate neuronal differentiation, thus providing insight into potential pharmacological therapies directed at endogenous NPCs.

Additionally, benzopyran-embedded tetracycle **29** has been found to have neuroprotective effects, specifically from the perspective of inhibiting microglia-mediated neuroinflammation [23]. Specifically, tetra-

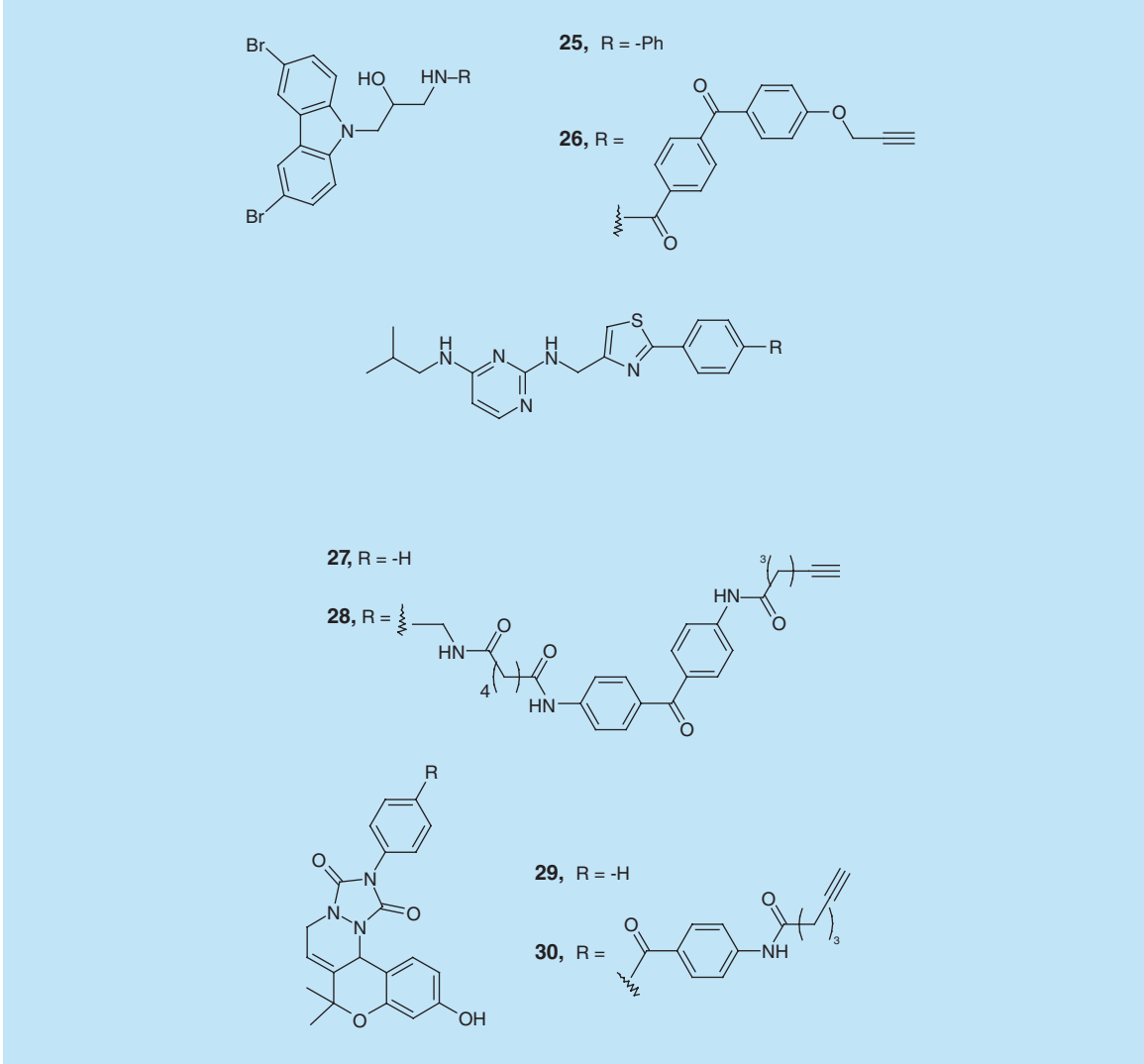

Figure 5. Lead compounds obtained from screening campaigns and their clickable benzophenone-based photoprobe derivatives for target identification.

cycle **29** was initially synthesized by privileged structure diversity-oriented synthesis (pDOS), and then discovered via cell-based phenotypic screening aimed at identifying novel anti-inflammatory agents. On the basis of SAR for related compounds, benzophenone-embedded photoprobe **30** was prepared and found to have inhibitory activity comparable to parent compound **29**. Subsequent tandem photoaffinity labeling-bioorthogonal conjugation, followed by fluorescence difference in two-dimensional (2D) gel electrophoresis (FITGE) technology [24], indicated HMGB2 as a potential target for chromene **29**. Indeed, further mode-of-action studies revealed that tetracycle **29** perturbs post-translational modification of HMGBs and reduces neuronal damage by downregulating the proinflammatory functions of these proteins. As a result, chromene **29** and its related analogs represent promising candidates for treating diseases associated with neuroinflammation.

Diazirine-based probes

Not only is drug resistance a major concern in cancer chemotherapy, this challenge is also apparent with antibiotics. In this regard, thioether **31** (Figure 6) was identified by high-throughput screening to suppress the expression of streptokinase, a bacterial activator of human plasmin that plays a direct role in enhancing the virulence of Group A Streptococci (GAS) [25]. Studies then proceeded toward target identification via the generation of multiple clickable photoprobes, highlighted by the placement of different photoreactive groups around the chemical scaffold [26]. Out of the seven probes synthesized and pharmacologically evaluated, which included aryl azide- and benzophenone-based probes, diazirine probe **32** maintained promising inhibition of streptokinase expression. Unfortunately, photoaffinity labeling with **32** in whole cells, followed by cell lysis and click chemistry with an azido fluorophore, revealed widespread fluorescence consistent with nonspecific labeling of a subset of GAS proteins. Currently, this group is pursuing probes with greater potency in the hopes of reducing nonspecific labeling.

In another study related to integration of phenotypic screening with target identification, a library of diazirine-based clickable photoprobes (**33**) was syn-

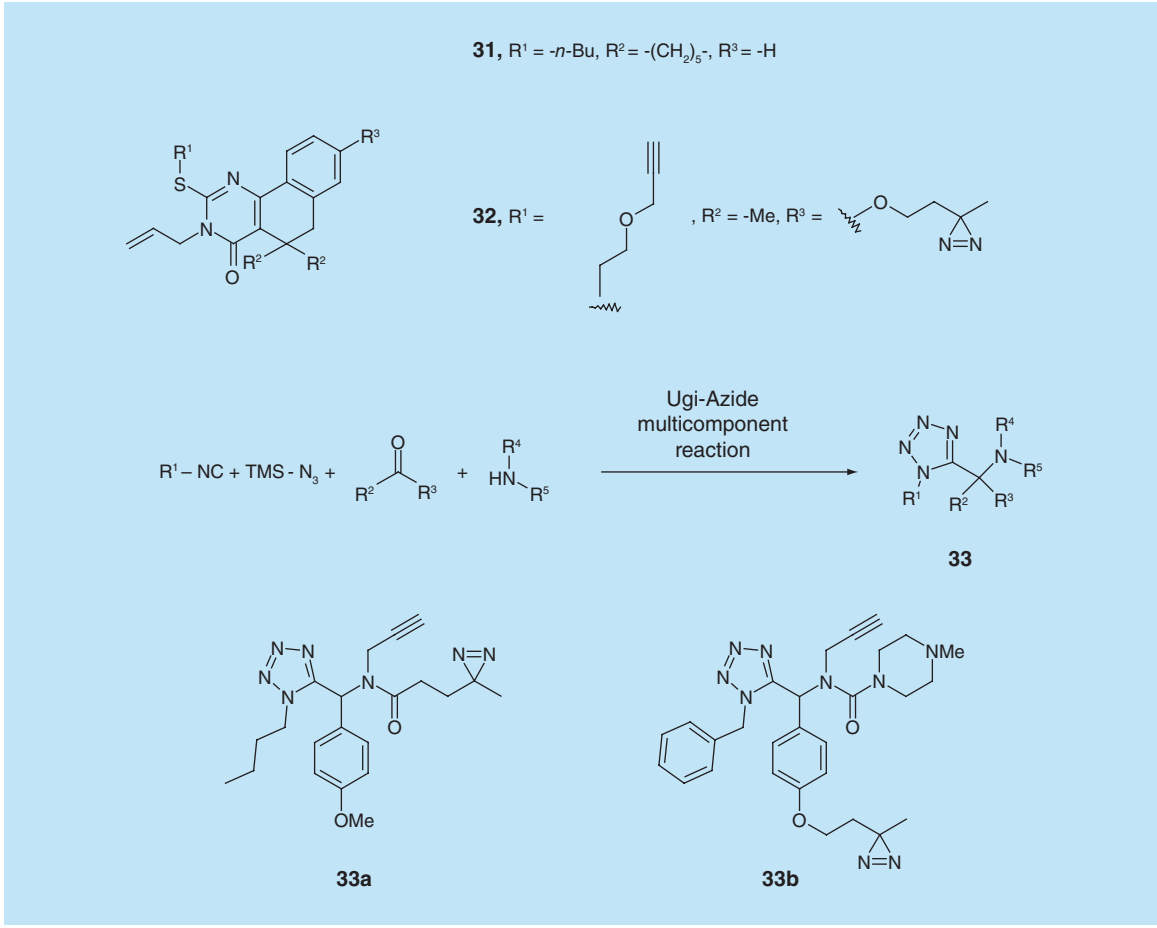

Figure 6. Diazirine-based clickable photoprobes for target identification of hit compounds arising via phenotypic screening.

thesized by multicomponent reaction technology [27]. Subsequently, the protein interaction landscape of these compounds was systematically examined in human cells. Representative examples of photoprobes from the 60-member library are alkynes **33a** and **33b**. In short, a diverse array of proteins, including adaptor and scaffolding proteins, enzymes and channels, were revealed upon photoaffinity labeling, click chemistry and MS-based analysis. Interestingly, lead ligands had not been described for many of the proteins found during this work. Furthermore, a significant number of probe–protein interactions showed well-defined SAR across the library and could be eliminated by small-molecule competitors in cells. In particular, this work highlights the promise of compounds bearing latent affinity handles and photoreactive groups as a fruitful source of pharmacological probes to survey diverse segments of any organism's proteome.

Clickable photoprobes for mechanism of action studies, confirmation of target engagement or selectivity profiling (also known as affinity-based protein profiling)

Activity-based protein profiling (ABPP) is an established powerful strategy for studying the activities of enzymes in their natural settings [4,28]. In particular, ABPP probes are typically substrate or inhibitor analog-bearing electrophiles that react irreversibly with a nucleophilic amino acid in an enzyme active site. Such probes then allow one to study the inhibitory effects of small molecules, discover new enzyme activities or determine the subcellular location of active enzymes. A specific class of ABPP probes called 'affinity-based probes' (AfBP, and hence, AfBPP) includes compounds that use a photoreactive group to create a covalent bond to their protein target(s) [4]. Such probes can target enzymes or other target classes that do not utilize a catalytic nucleophilic amino acid for covalent bond formation, but instead will rely on photoaffinity labeling to form an irreversible probe–protein complex. In principle, photoaffinity probes do not necessarily label active enzymes. However, compounds can be rationally designed such that an active site-directed photoprobe specifically binds to the active form of the enzyme prior to covalent bond formation via photoirradiation (e.g., transition state analog γ-secretase inhibitor photoprobes that only label the active γ-secretase complex, *vide infra*). In this regard, photoaffinity labeling and ABPP frequently go hand in hand.

Besides using clickable photoprobes for selectivity profiling/AfBPP, such compounds can also be used to confirm target engagement in live cells [29] and aid in mechanism of action studies. The following are examples of clickable photoprobes used for such applications.

Natural products

Polymyxins are macrocyclic antibiotics used to treat serious infections caused by multidrug-resistant Gram-negative bacteria. However, the mechanism of action of these natural products produced by the *Bacillus* species of bacteria remains incompletely understood. In order to detect interactions between outer membrane proteins and polymyxin B3 (**34**, Figure 7), previous SAR studies led to the rational design and synthesis of diazirine-based clickable photoprobe **35**, which features L-photoleucine replacement of a L-Leu residue and addition of an oct-7-ynol group to the N-terminus of the peptide as a click chemistry handle [30]. Upon pharmacological evaluation, photoprobe **35** retained potent antimicrobial activity, and initial photoaffinity labeling experiments using *Escherichia coli* ATCC25922 and biotin azide indicated probe labeling of several outer membrane proteins. As a result, photoprobe **35** could represent a valuable compound for elucidating the mechanism of action of polymyxin antibiotics.

With respect to vancomycin, the Sieber group utilized clickable benzophenone photoprobe **36** to identify two previously unknown protein targets for vancomycin in living bacterial cells, namely autolysin Atl and an ABC transporter protein [31]. The labeling of these two prominent membrane targets in living cells highlights the power of the clickable photoprobe approach, since it would have been extremely challenging to pull these targets down using a traditional affinity chromatography approach. In addition to the photoreactive groups highlighted thus far, pyrimidones are well-documented photoreactive moieties that are found in a number of different natural products and bioactive small molecules (e.g., zebularine, a DNA methylation inhibitor). In particular, pyrimidones (**38**, Figure 7) can undergo a Norrish type I reaction to form a bicyclic intermediate (**39**) or an isocyanate (**40**), which can subsequently react with nucleophiles via affinity labeling to form a covalent adduct (**41**) [32,33]. Such a process may be referred to as photomasked affinity labeling. As a proof-of-concept experiment, vancomycin-based pyrimidone probe **37** was shown to selectively label the proteins pABC and ATLam in *E. coli* identical to that of vancomycin-based benzophenone probe **36** [34]. Such a result indicates that pyrimidones are intrinsically photoreactive natural functional groups that may find appropriate utility in future AfBPP probes.

Related to the antibiotics polymyxin B and vancomycin is research in the area of bacterial quorum sensing. Bacterial quorum sensing is a mechanism that enables bacteria to regulate their gene expression in response to alterations in cell population density. This mechanism can ultimately control a number of

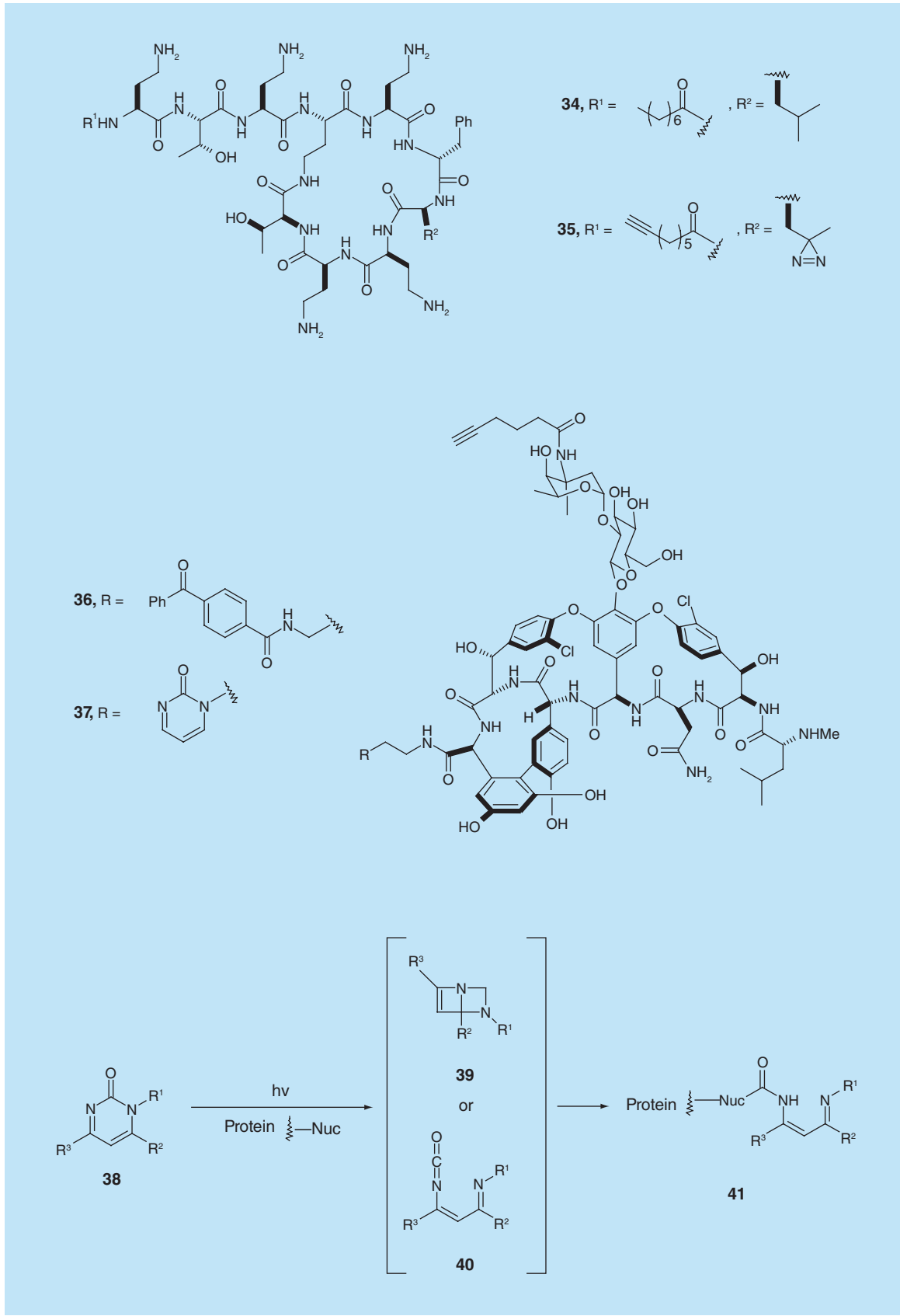

Figure 7. Clickable antibiotic photoprobes.

important properties associated with bacteria including their motility, biofilm formation, virulence factor expression and bioluminescence. In particular, the main compounds that facilitate bacterial quorum sensing in Gram-negative bacteria are *N*-acyl homoserine lactones (e.g., **42**, Figure 8). In this regard, several clickable aliphatic diazirine photoprobes (**43**) were designed and synthesized toward potentially identifying and isolating receptors for *N*-acyl homoserine lactones in different species [35]. In short, it was observed that low micromolar concentrations of photoprobes **43a** and **43b** were excellent mimics of **42** in binding and functional assays. However, probe **43c** performed surprisingly poor in these same assays, thus indicating the importance of the position of the photoreactive diazirine along the lipid chain.

In the area of natural products with anticancer activity, aplyronine A (**44**, ApA, Figure 8) is an apoptogenic and antitumor macrolide with potent actin-depolymerizing activity. Under the premise that the noteworthy anticancer activities of macrolide **44** may not solely be due to its interactions with actin, aryl diazirine clickable photoprobe **45** was designed and synthesized toward potentially finding additional biological targets associated with aplyronine A [36]. Diazirine **45** showed potent cytotoxicity against HeLa S3 cells comparable to that of parent **44**, and was shown to form a covalent bond to actin via tandem photoaffinity labeling-bioorthogonal conjugation. However, this work also featured biotinylated photoaffinity derivatives of **44**, which were used to identify target proteins of aplyronines in tumor cell lysates. Specifically, Arp2 and Arp3 were purified as binding proteins, along with actin, from a tumor cell lysate using biotinylated photoreactive derivatives of aplyronine A and C. This work suggests that actin-related proteins might indirectly bind to ApA through oligomeric actin, or as the ternary adduct of an actin/ApA complex.

Finally, penicillenol C_1 (**46**, Figure 8) is a natural product that can be isolated from the *Penicillium* fungal species GQ-7. However, there are no data concerning the distribution or uptake by cells of this natural product. Furthermore, the compound has only been tested for biological activity to a limited extent despite being closely related to the well-known melophilins. As part of a total synthesis effort of penicillenol C_1, bis-azide tagged derivative **47** was generated as a clickable photoprobe toward eventually identifying the cellular targets of the penicillenol family of natural products [37]. Specifically, photoprobe **47** features an aromatic azide, which acts as the photoreactive group to covalently modify target proteins. In contrast, the aliphatic azide survives the specific photolysis conditions and can be used as a click chemistry handle following photoaffinity labeling. As one part of this work, the distribution of diazide **47** was tracked in PTK2 kidney cells via tandem photoaffinity labeling-Staudinger-Bertozzi ligation featuring a fluorophore–phosphine conjugate.

Known drugs & promising clinical candidates

Praziquantel (**48**, Figure 9) is a widely available drug for the treatment of schistosomiasis, a pervasive tropical disease caused by blood-dwelling trematode worms. However, the mode of action of praziquantel remains poorly understood, particularly the identity of its biological target(s). Toward addressing this research question, several molecular probes of praziquantel were rationally designed and prepared, including fluorescent and clickable photoprobe derivatives (**49**) [38]. However, it was found upon biological evaluation that photoprobes **49** did not kill schistosomes, even at relatively high concentrations. The authors speculate that the increased molecular weight and lipophilicity do not allow these compounds to readily cross the worm tegument, thus making them significantly less active and effective versus praziquantel. As a result, additional probes and studies are currently in progress toward overcoming this problem.

In a similar light in the area of infectious disease, albitiazolium (**50**, Figure 9) is a clinical candidate for the treatment of malaria that was designed from a series of choline analogs to disrupt *Plasmodium falciparum* phospholipid metabolism. Toward identifying proteins targeted by this compound in living parasites, a bis-azide probe similar to penicillenol C_1 derivative **47** was pursued, except probe **51** features a benzyl ether linkage instead of a benzamide [39]. In particular, tandem photoaffinity labeling-bioorthogonal conjugation, followed by MS and clustering of gene ontology terms, uncovered parasite proteins involved in lipid metabolism, transport and binding as potential drug targets for albitiazolium. Furthermore, it was discovered that probe **51** was localized in the trans-Golgi network and endoplasmic reticulum of *P. falciparum*. Finally, competitive binding assays involving choline/ethanolamine phosphotransferase, the enzyme that catalyzes the last step of phosphatidylcholine biosynthesis in *P. falciparum*, were also used to support the search for albitiazolium drug targets during the course of this work.

PD-404182 (**52**, Figure 9) is a potent antiviral agent against hepatitis C virus and human immunodeficiency virus (HIV). However, much like praziquantel and albitiazolium, the protein targets of PD-404182 remain undetermined. In order to investigate the mechanism of action of PD-404182, three

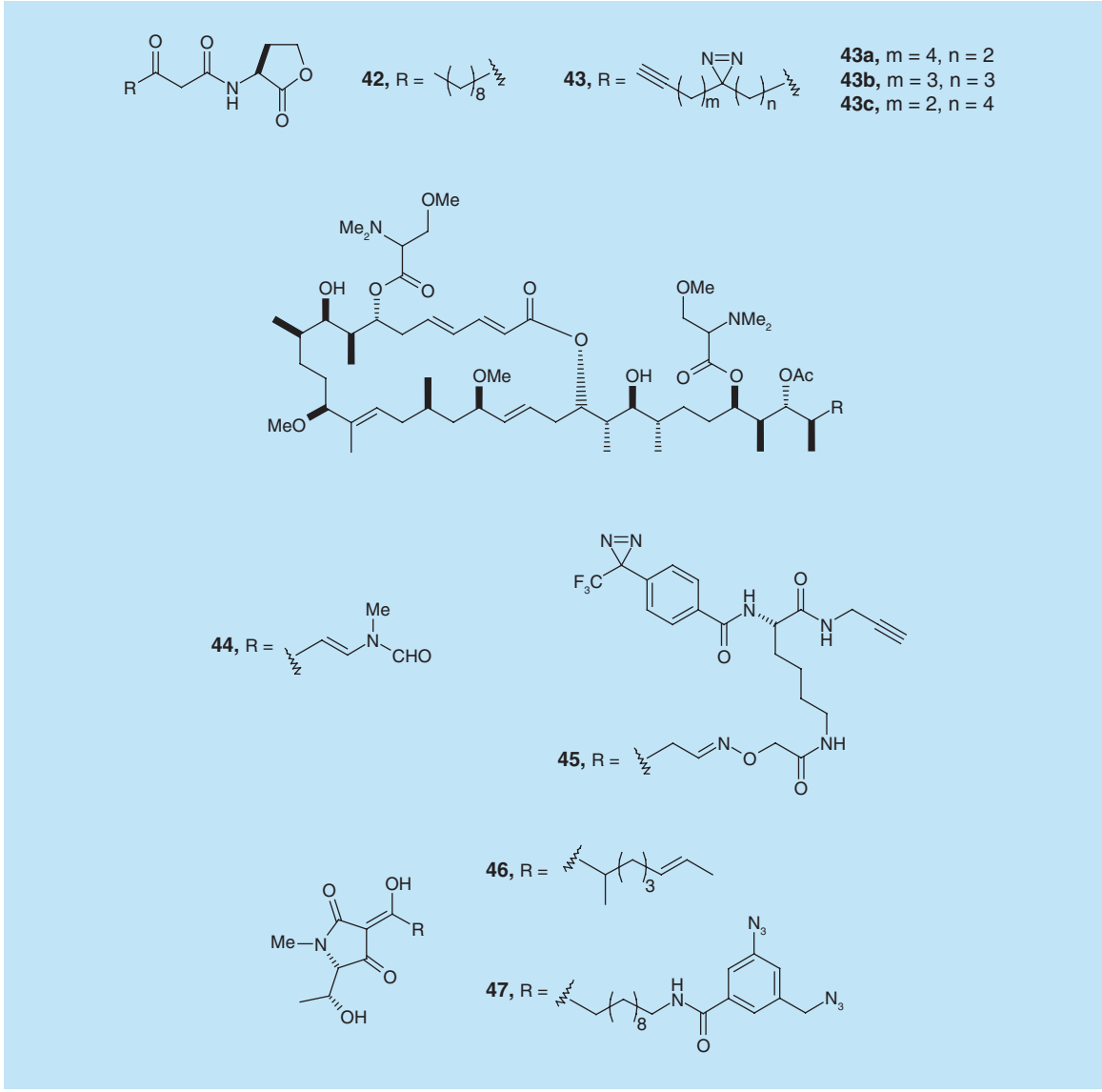

Figure 8. Additional examples of clickable photoprobes based on natural products.

photoaffinity analogs of this promising antiviral candidate were designed to include either a biotin affinity handle or an alkyne click chemistry handle [40]. In particular, clickable photoprobe **53** bearing a photoreactive benzophenone potently inhibited HIV infection comparable to that of PD-404182, whereas biotinylated derivatives resulted in a >11-fold loss in activity. Despite this observation, photoaffinity labeling experiments were only performed with the biotinylated analogs, and eight potential proteins were found to bind PD-404182 in HIV-1-infected H9 cells. The authors state photoaffinity labeling experiments are currently in progress with clickable photoprobe **53**.

As one final example of a promising clinical candidate in the area of infectious disease, Sal-AMS (**54**, Figure 9) is a potent antitubercular agent that functions as a bisubstrate inhibitor of the adenylating enzyme MbtA. The finding that Sal-AMA inhibits MbtA under both iron-deficient and iron-replete growth conditions is somewhat unusual. This is because mycobactins, which are biosynthesized via MbtA, are not required for the growth of *Mycobacterium tuberculosis* under iron-replete conditions. As a result of the previous observations, it is possible Sal-AMS may potentially inhibit additional biochemical pathways as an antitubercular clinical candidate. With this research question in mind, clickable photoprobe **55** was designed and shown to photolabel MbtA in mycobacterial lysate, as well as the pure enzyme [41]. In particular, the prototypical core scaffold associated with photoprobe **55** may lead to the creation of future ABPP probes for profiling adenylating enzymes in pathogenic bacteria.

Alternative to infectious disease, clickable photoprobes for clinical candidates in other disease state

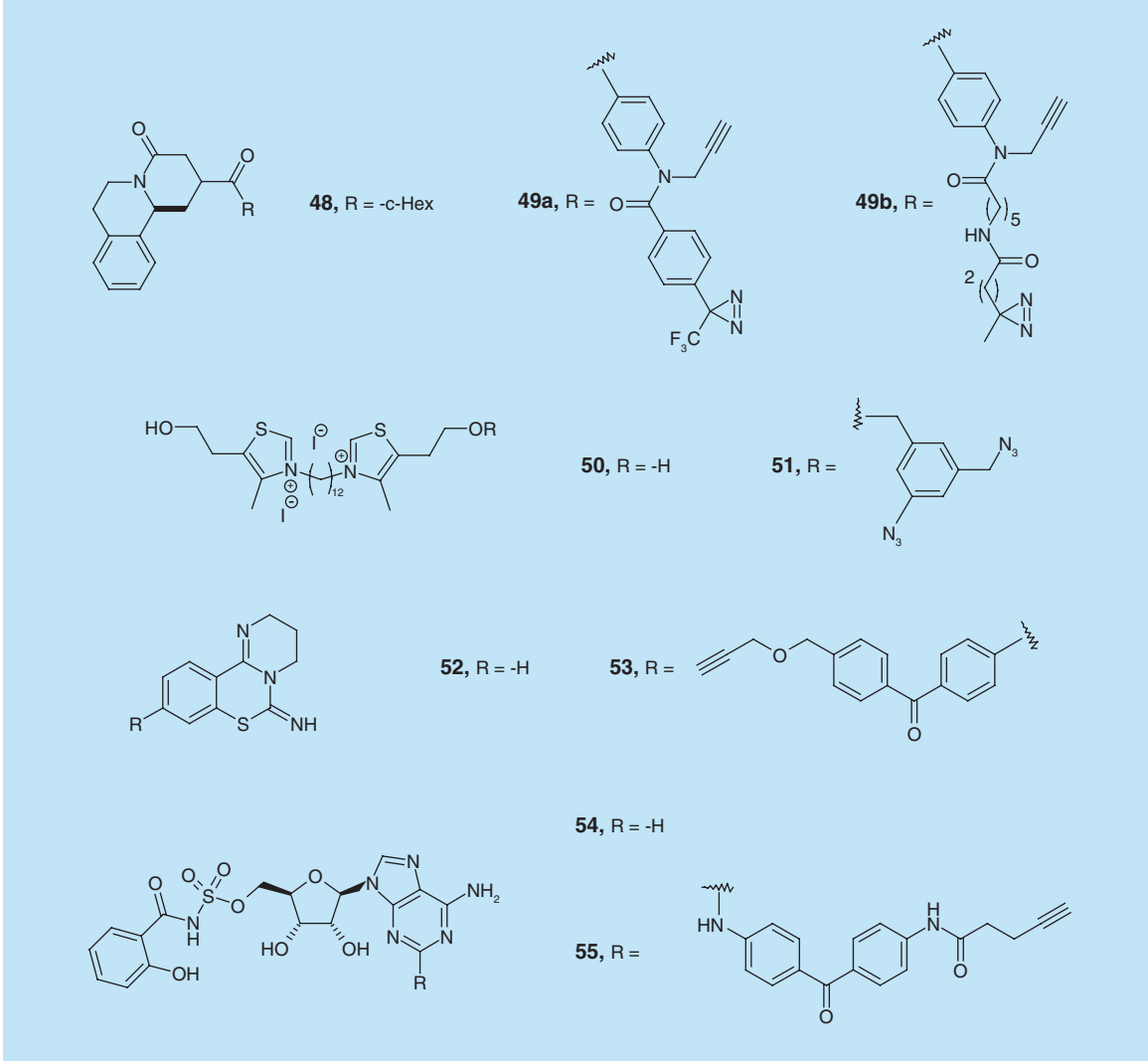

Figure 9. Clickable photoprobes based on known drugs and promising clinical candidates in the area of infectious disease.

areas have been reported (Figure 10). For example, bis(trifluoromethyl)pyrazole derivative YM-58483 (**56**) is a clinical candidate for bronchial asthma, transplant rejection and cardiac hypertrophy, whereas MP-10 (**58**) is a phophodiesterase 10A (PDE10A) inhibitor clinically being considered for Huntington's disease, Parkinson's disease and schizophrenia. Specifically, employment of clickable diazirine **57** in photoaffinity labeling revealed direct binding of YM-58483 to transient receptor potential channel 3 [42]. This particular example of tandem photoaffinity labeling-bioorthogonal conjugation is rather unique, as it is the only example to date that features a ketone functional group as a bioorthogonal chemistry handle and employment of a biotin hydroxyl-amine reagent for oxime click chemistry (Figure 3B) after photoaffinity labeling. In contrast, benzophenone-based clickable photoprobe **59** was used to measure target engagement and characterize the protein profile of MP-10 in both membrane and whole cell preparations [43]. Interestingly, biotinylated affinity probes also pursued during the course of this work revealed exquisite binding of MP-10 to PDE10A with essentially no off-target binding.

Key term

Bioorthogonal chemistry: Refers to any chemical reaction that can take place inside a living system that does not impede the native biochemical processes associated with that system. Typical requirements for a chemical reaction to be classified as bioorthogonal are chemoselectivity, chemical and biological inertness and compatibility, and fast reaction kinetics. While a number of chemical reactions meet these criteria, the 1,3-dipolar cycloaddition of azides with cyclo-octynes (also known as strain-promoted azide alkyne cycloaddition [SPAAC]) and the inverse electron demand Diels-Alder (iEDDA) reaction of tetrazines with strained alkenes, represent two of the most common bioorthogonal reactions.

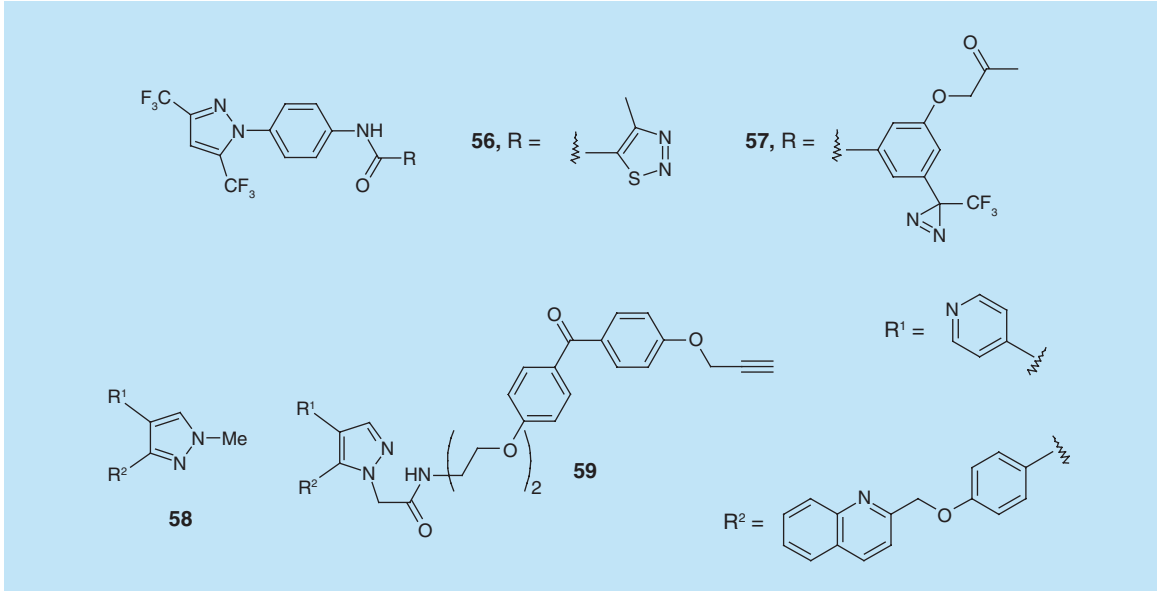

Figure 10. Clickable photoprobes for clinical candidates in disease states alternative to infectious disease.

Kinases

Protein kinases are key enzymes that regulate cell function by transferring phosphate groups to specific substrates. In particular, the phosphorylation state of a biological molecule (e.g., carbohydrates, lipids and proteins) can influence its ability to bind to other molecules, reactivity and activity. As a result, kinases represent tremendously important drug targets in industry and academics given their ability to control cell signaling, metabolism, protein regulation and many other cellular processes. Additionally, a number of research groups continue to be involved in the development of clickable photoprobes based on kinase inhibitors for a variety of applications (Figure 11).

In order to minimize steric interference upon photoprobe binding to target proteins, a set of 'minimalist' clickable photoreactive reagents, each containing an aliphatic diazirine and a terminal alkyne, were recently designed and attached to 12 different kinase inhibitors for tissue- and cell-based proteome profiling [44]. Probes **60** and **61** represent selected examples from the initial report of these reagents. In short, probes that contained 'minimalist' linkers performed significantly better in proteome profiling cellular kinase targets compared with previous analogs that employed a larger *N*-alkynylated L-photoleucine unit. As an extension of this work, diazirine **62** represents the first photoaffinity-based Aurora kinase A (AKA) probe capable of *in situ* proteome profiling of potential off-targets of MLN8237 (i.e., a presumed selective and highly potent inhibitor of AKA) and live-cell imaging of AKA activities [45].

Alternative to aliphatic diazirines, aromatic diazirines were recently applied to type II kinase inhibitors that stabilize a specific inactive shape of the ATP-binding site known as the DFG-out conformation. In particular, employment of the conformation-selective properties associated with photoprobes **63** and **64** in AfBPP aided in finding a number of protein kinases not previously characterized to adopt the DFG-out conformation [46]. This same research group has also developed a set of label transfer compounds to identify proteins involved in p38 mitogen-activated protein kinase signaling complexes. One example from this work is clickable benzophenone photoprobe **65** [47]. Specifically, clickable photoprobes generated during the course of this study were cell permeable and not only identified p38 substrates, but repressors, activators and other binding partners as well.

As final examples in the area of kinases, benzophenone-embedded **67** was the most potent compound in a series of clickable photoprobes aimed at identifying the molecular target of CCG-1423 (**66**), an Rho/MKL1/SRF signaling pathway inhibitor [48]. Specifically, tandem photoaffinity labeling-bioorthogonal conjugation employing photoprobe **67** in intact PC-3 cells indicated specific labeling of a single 24-kDa protein, whose identity is currently being determined. Additionally, PIK-BPyne (**69**) was reported as a new chemical probe for profiling phosphatidylinositol kinase (PIK) activity in native biological systems [49]. In particular, benzophenone **69** stemmed from lipid kinase inhibitor PIK-93 (**68**) and proved comparable to a previously reported wortmannin activity-based probe known to target PIKs.

HIF-1α

HIF-1 is a complex transcription factor responsible for regulating tumor growth in response to oxygen

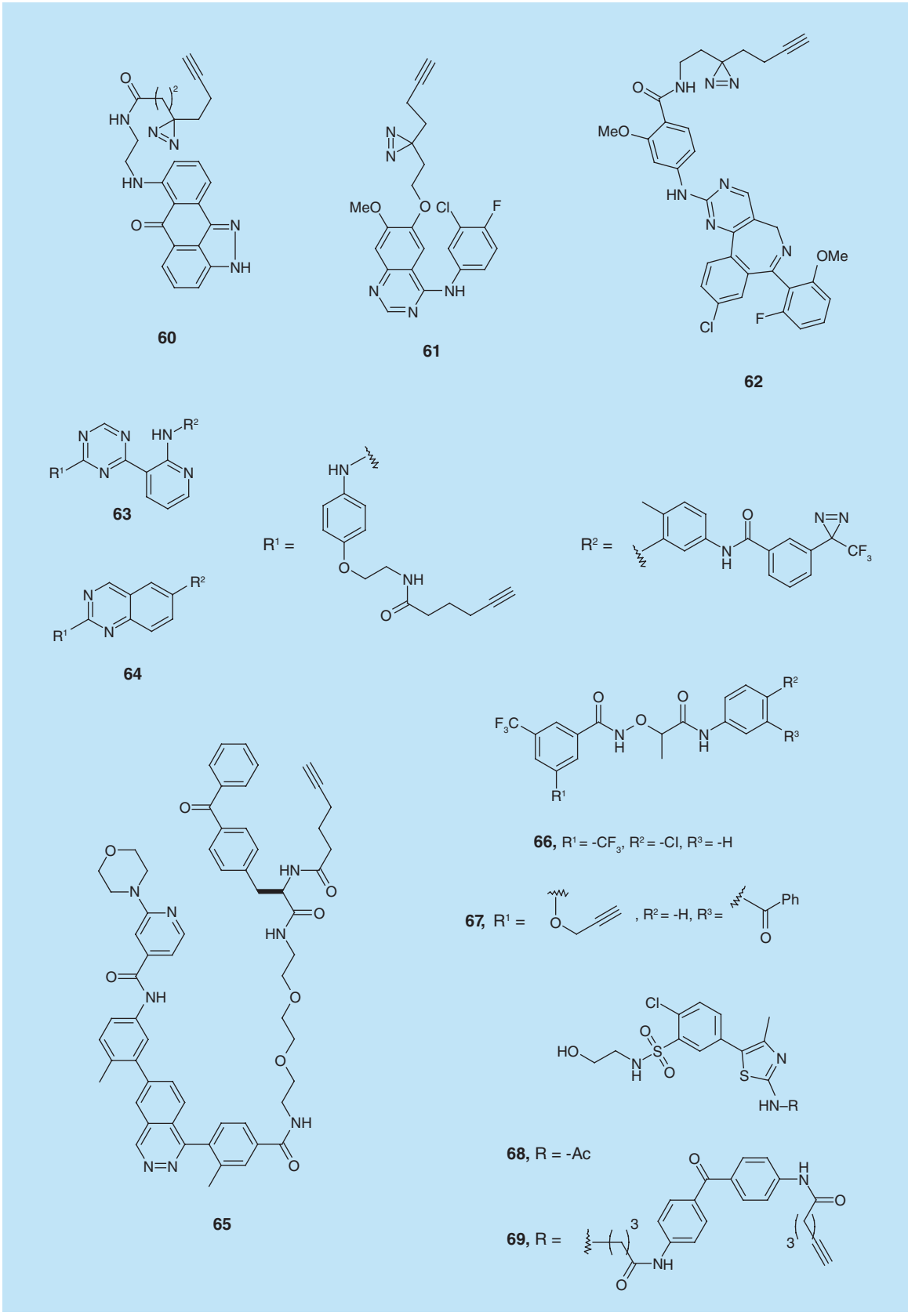

Figure 11. Clickable photoprobes based on kinase inhibitors.

deficiency. In particular, high levels of HIF-1α, one component of the HIF-1 heterodimer, have been correlated with poor clinical outcomes via aggressive tumor growth, and resistance to chemotherapy and radiation. As a result, the pursuit of clinically effective HIF-1α inhibitors has attracted notable attention in recent years.

One promising HIF-1α inhibitor is aryloxyacetylamino benzoic acid derivative LW6 (**70**, Figure 12), which under hypoxic conditions potently inhibits HIF-1α accumulation and target gene expression. In order to better understand the mechanism of action of LW6, an SAR study for a series of multifunctional chemical probes was pursued with the intention of identifying compounds to determine the biological targets of this lead compound [50]. From this work, diazirine alkyne **71** emerged as a promising candidate that displayed strong inhibitory activity against HIF-1α. Subsequent tandem photoaffinity labeling-bioorthogonal conjugation using clickable photoprobe **71** led to the identification of MDH2 as a target protein for LW6, which was further confirmed via an MDH2 enzyme assay [51]. Additionally, competitive binding modes of diazirine **71** and LW6 to MDH2 was demonstrated [50]. In a follow-up study associated with tumor angiogenesis, reverse chemical proteomics in live cells using diazirine **71** also identified CHP1 as a target for LW6 [52]. Subsequent experiments further demonstrated LW6 inhibits HIF-1α stability by direct binding to CHP1, which subsequently results in the suppression of angiogenesis. As a result, photoprobe **71** represents a valuable tool compound toward understanding how CHP1 and MDH2 regulate HIF-1 function.

Alternative to LW6, carboranylphenoxyacetanilide GN26361 (**72**, Figure 12) is also known to induce HIF-1α degradation under oxygen-deficient conditions. Toward clarifying the mechanism of **72** against HIF inhibition, the phenol group was removed and the boronic acid was replaced to create an embedded benzophenone alkyne (**73**) for target identification studies [53]. Subsequent tandem photoaffinity labeling-bioorthogonal conjugation identified heat shock protein 60 (HSP60) as a target protein for GN26361. Although HSP90 is well known to stabilize HIF-1α under hypoxic conditions, this work demonstrates that HSP60 also plays an important role in this process.

γ-secretase

A particularly challenging target for drug development is γ-secretase, which is an aspartyl protease complex composed of at least four subunits: presenilin, Pen-2, Aph-1 and nicastrin. The most widely known substrate of γ-secretase is APP, which when cleaved by both β- and γ-secretase produces amyloid-β peptides (Aβ). In particular, the abnormally folded fibrillar form of Aβ is the primary constituent of amyloid plaques, which are routinely found in the brains of patients with Alzheimer's disease. As a result, modulators and inhibitors of γ-secretase are currently being pursued for the treatment of Alzheimer's disease.

Over the past several years, a number of clickable photoprobes have been reported toward understanding ligand-binding sites within γ-secretase (Figure 13). For example, piperidine acetic acid γ-secretase modulator GSM-1 (**74**) has been shown to directly bind to the N-terminal fragment of presenilin-1 (PS1-NTF) via photoaffinity labeling studies using azide-based clickable photoprobe GSM-5 (**75**) [54]. Likewise, imidazole γ-secretase modulator E2012 (**76**) and γ-secretase inhibitor BMS-708,163 (**78**) are also known to directly

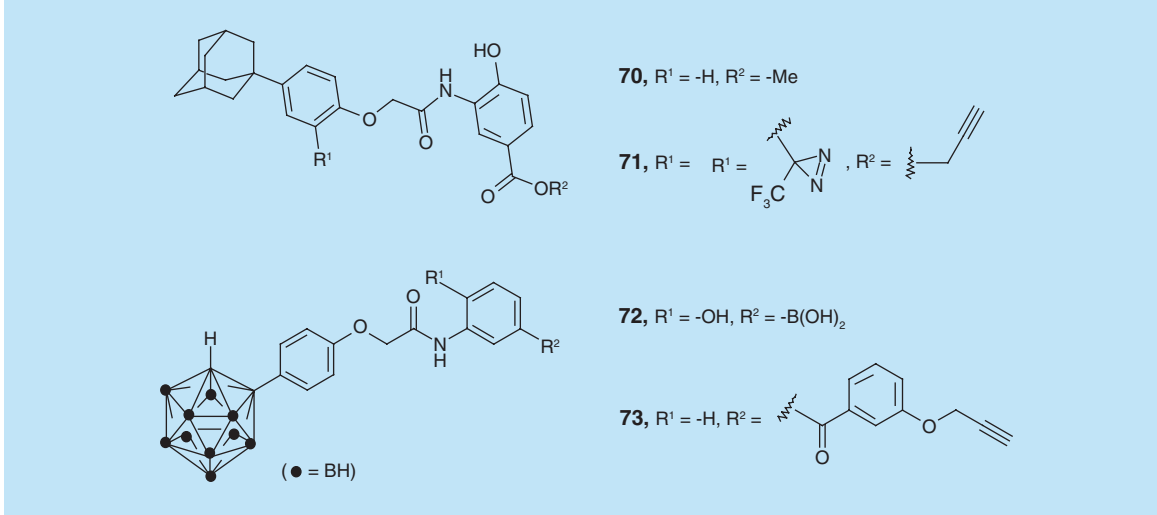

Figure 12. Clickable photoprobes associated with HIF-1α.

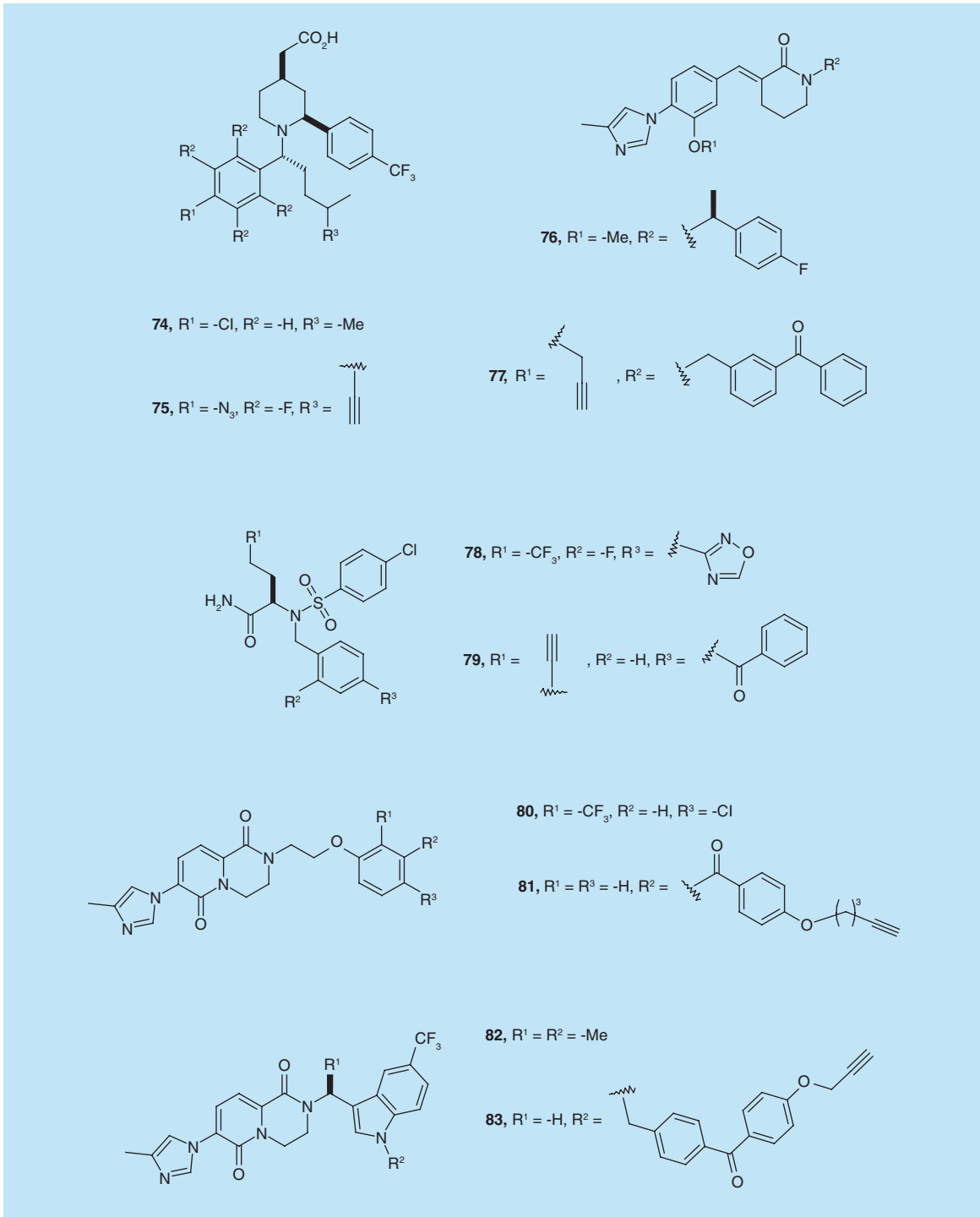

Figure 13. Clickable photoprobes toward understanding ligand-binding sites within γ-secretase.

bind to PS1-NTF via similar labeling studies employing clickable photoprobes E2012-BPyne (**77**) [55] and 163-BPyne (**79**) [56]. However, the inability of allosteric γ-secretase inhibitor BMS-708,163 (**78**) and γ-secretase modulator GSM-1 (**74**) to effectively block photolabeling of E2012-BPyne (**77**) to PS1-NTF, combined with the inability of BMS-708,163 (**78**) and E2012 (**76**) to block specific photolabeling of clickable photoprobe GSM-5 (**75**) to PS1-NTF, suggests E2012 (**76**) occupies a different binding site on PS1-NTF that is distinct from the binding site for γ-secretase inhibitor BMS-708,163 (**78**) and γ-secretase modulator GSM-1 (**74**) [55]. In addition, it was shown that binding of the active site-directed γ-secretase inhibitor L458

(**84**, Figure 14) enhanced the photolabeling of E2012-BPyne (**77**), providing evidence that there is crosstalk between the active site and the E2012 (**76**) allosteric binding site. Additionally, optimized pyridopyrazine-1,6-dione γ-secretase modulators **80** and **82** have also been shown to target PS1-NTF via tandem photoaffinity labeling-bioorthogonal conjugation involving their corresponding clickable photoprobes **81** [57] and **83** [58]. Collectively, these clickable photoprobes suggest that multiple binding sites exist within γ-secretase, and a variety of experimental data indicates that each of these sites may furnish different modes of γ-secretase modulation upon binding of a specific ligand.

Alternative to trying to understand ligand-binding sites in γ-secretase, a suite of clickable photoprobes (**85**, Figure 14) were designed and synthesized with the aim of identifying proteins that may interact with γ-secretase in the proximity of its active site [59]. For this work, probe design began with active site-directed γ-secretase inhibitor L458 (**84**), which was subsequently derivatized with two photoreactive benzophenone groups – one proximal to the inhibitor scaffold (i.e., R^1) that is expected to form a covalent bond within the γ-secretase active site, and the other (i.e., R^2) made remote from the active site by means of a variable length linker attached to the inhibitor for the purpose of surveying the γ-secretase microenvironment/interactome. In brief, tandem photoaffinity labeling-bioorthogonal chemistry using probes **85** resulted in specific labeling of PS1-NTF, the C-terminal fragment of presenilin-1 (PS1-CTF) and nicastrin, a protein not directly at the active site of γ-secretase. Additionally, these probes provided strong evidence of a protein crosslink between PS1-CTF and PS1-NTF resulting in the formation of a pseudo-full length presenilin-1.

Finally, γ-secretase undergoes endoproteolysis of its catalytic subunit, presenilin, to form both presenilin C-terminal and N-terminal fragments, which give rise to the active site. The enzyme responsible for the endoproteolysis of presenilin is known as presenilinase, which alternative to Alzheimer's disease has been suggested as a drug target for cancer. In particular, CBAP (**86**, Figure 14) is a rather unique compound that functions as a dual γ-secretase and presenilinase inhibitor, subsequently causing accumulation of full-length presenilin-1 in cells while pharmacologically knocking down PS1-NTF and PS1-CTF. However, the mechanism of action of CBAP with respect to this dual inhibition is not understood. In order to address this knowledge gap, clickable photoprobe CBAP-BPyne (**87**) was designed and shown to profile γ-secretase and presenilinase activity in cells [60]. In particular, CBAP-BPyne proved to specifically label signal peptide peptidase and PS1-NTF upon tandem photoaffinity labeling-bioorthogonal conjugation. Future studies associated with this probe will move toward identifying and characterizing presenilinase in order to understand the mechanism of γ-secretase activation.

Epigenetic drug targets

Epigenetics refers to functionally relevant changes in gene expression without a change in underlying DNA sequence (i.e., activation versus inactivation of genes resulting in a change in phenotype without a change in genotype). In particular, certain epigenetic changes can have very damaging effects that result in disease states such as cancer and Alzheimer's disease. At least three systems have been linked to initiating and sustaining epigenetic change: histone modification, DNA methylation and noncoding RNA-associated gene silencing. In particular, several groups have developed clickable photoprobes toward better understanding a number of enzymes and proteins currently being considered as epigenetic drug targets (Figure 15).

Histone deacetylases (HDACs) are a class of enzymes that remove acetyl groups from the side chains of lysine residues within histone proteins that have been acetylated. As a result of this deacetylation, DNA can wrap itself around histone proteins more tightly, thus affecting DNA expression. In turn, the link of HDACs to epigenetics has led a number of groups to pursue the development of inhibitors of these enzymes as potential therapeutics. In this regard, a series of diazide-based clickable photoprobes based on novel HDAC8 ligands that do not contain a zinc-chelating group were developed in order to better understand the binding mode of these compounds [61]. In particular, photoprobes **88b**–**88e** were found to be likely positioned upside-down (i.e., relative to parent compound **88a**, which contains a hydroxamic acid zinc-chelating group) in a secondary binding site adjacent to HDAC8's catalytic site. Analogously, bis-azide **89** and its analogs were employed as nanorulers for determining the distance between the deacetylase activating domain of the silencing mediator for retinoid or thyroid hormone receptors (SMRT-DAD) and the catalytic site of full-length HDAC3, wherein direct interaction of these two proteins is required for activation of HDAC3 enzymatic activity [62]. In particular, this work represents the first time clickable photoprobes were used to detect important conformational changes associated with a transcription complex upon response to chemical activation. Collectively, this work associated with HDACs 3 and 8 represent important contributions toward better understanding manipulation of the histone code at the molecular level.

In contrast to HDACs, lysine acetyltransferases (KATs) represent another key class of enzymes that are

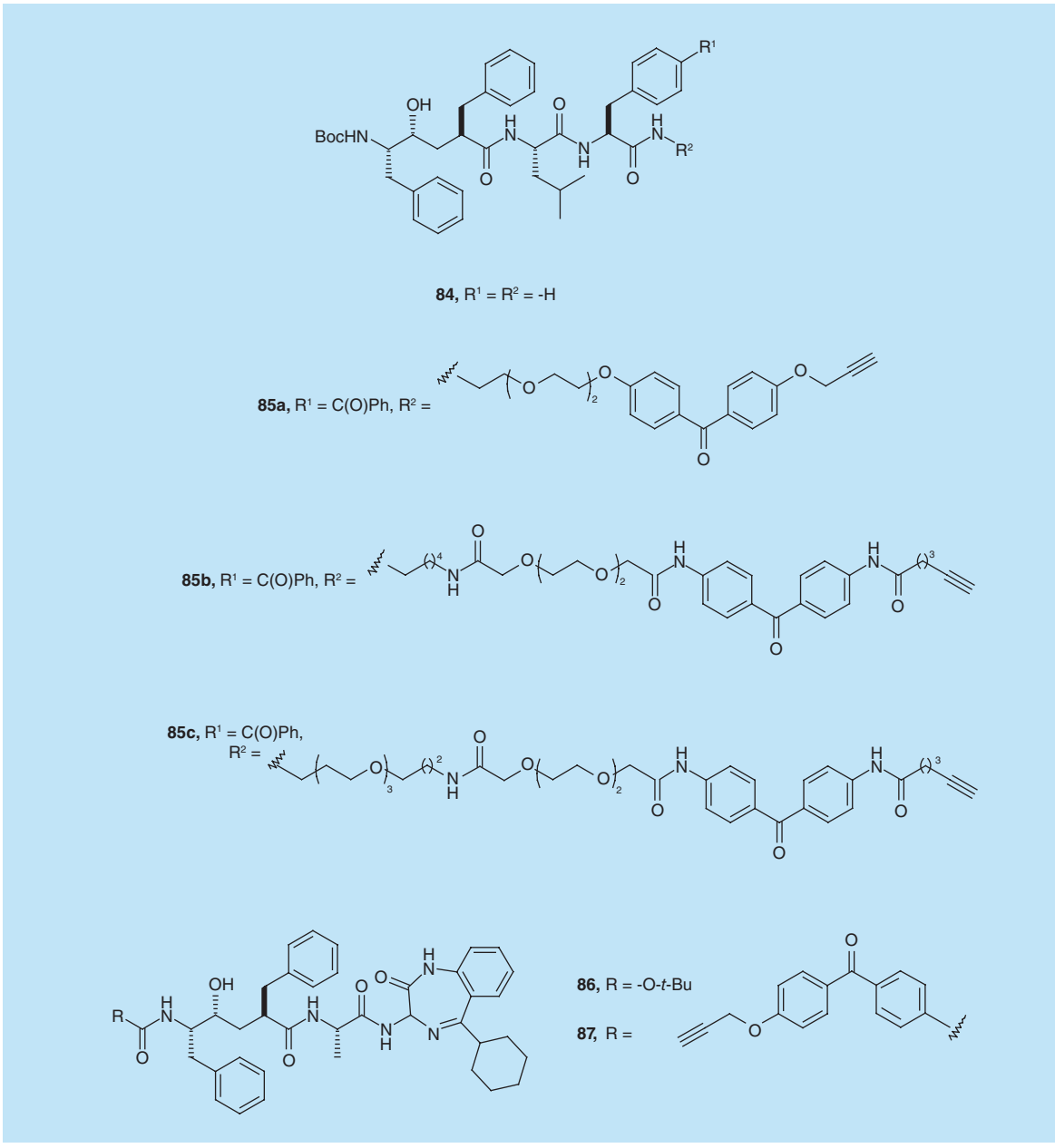

Figure 14. Clickable photoprobes toward investigating the γ-secretase interactome and understanding presenilinase.

involved in the regulation of metabolism, transcription and other biologically significant processes. In particular, KATs catalyze the acetylation of select lysine residues within certain proteins (e.g., histones) as an important post-translational modification. Currently, there are 18 known KATs in the human genome, and in particular, select KATs are known to fuel cancer progression in a tissue-specific manner. In order to facilitate future KAT inhibitor development, as well as the discovery and characterization of new KAT enzymes, a suite of benzophenone-based clickable photoprobes (e.g., **90**) based on KAT cofactors were synthesized and found to act as ABPP probes in cell lysates [63]. Additionally during this work, bisubstrate inhibitors based on three distinct KAT families were transformed into clickable photoprobes and shown to label and detect KAT as well.

Apart from acetylation and deacetylation, methylation of histone proteins represents another important post-translational modification in epigenetics. In this regard, 3-deazaneplanocin A (**91**), a carbocyclic analog of adenosine, has attracted noteworthy attention as a global histone methylation inhibitor for the potential treatment of cancer. However, the cellular targets of

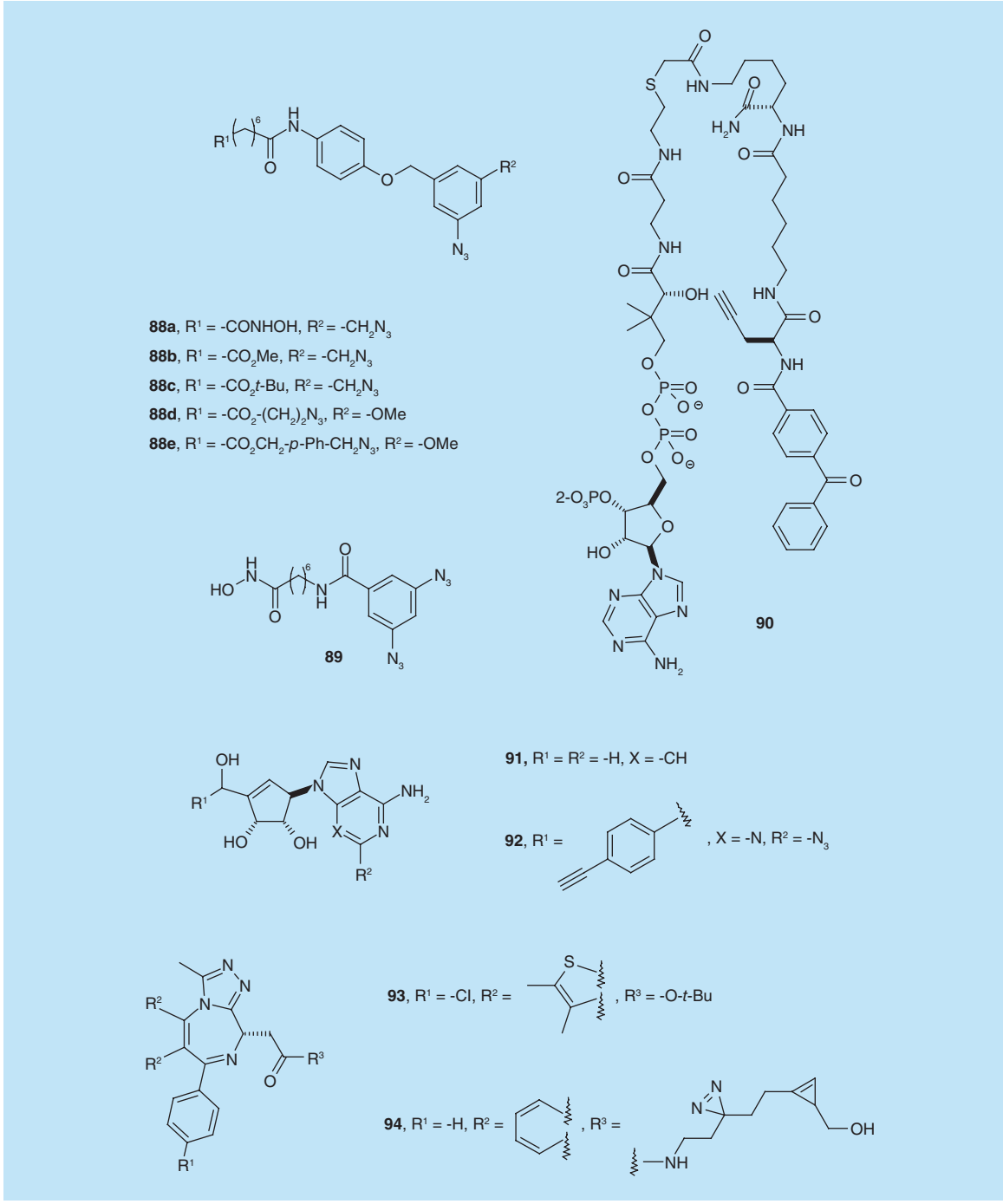

Figure 15. Clickable photoprobes associated with epigenetic drug targets.

this nucleoside analog remain incompletely characterized. In order to address this knowledge gap, azide-based clickable photoprobe DZ-1 (**92**) was rationally designed as an analog of 3-deazaneplanocin A, synthesized and found to possess similar anti-apoptotic activities comparable to **91** in MCF-7 mammalian cells [64]. Upon subsequent AfBPP in live mammalian cells via tandem photoaffinity labeling-bioorthogonal conjugation, LC–MS/MS results indicated a number of highly enriched proteins previously unknown to interact with 3-deazaneplanocin A. However, further experimentation is needed to confirm these proteins as either on- or off-targets associated with 3-deazaneplanocin A biological activity.

As a final example in the area of epigenetics, (+)-JQ1 (**93**) is a nanomolar protein–protein interaction inhibitor of bromodomain-containing protein-4 (BRD-4). In particular, BRD-4 contains two bromodomains,

which are ~s110 amino acid sequences that recognize monoacetylated lysine residues typically found at the N-terminal tails of histones. In general, the recognition of bromodomains with acetylated lysines in epigenetics is associated with chromatin remodeling and protein-histone association. Furthermore, BRD-4 is often required for expression of a number of tumor driving oncogenes in cancers such as acute myelogenous leukemia and multiple myeloma. With the goal of developing strategies for simultaneous bioimaging and target identification in live mammalian cells, principally via photoaffinity labeling coupled with copper-free bioorthogonal chemistry, (+)-JQ1 analog **94** was designed by attaching a minimalist moiety containing a cyclopropene for rapid tetrazine ligation (Figure 3B) and an aliphatic diazirine photoreactive group for covalent bond formation [65]. In turn, photoprobe **94** was employed in cell-based AfBPP, which resulted in the identification of several new off-targets for (+)-JQ1 that were subsequently validated during the course of this work.

Miscellaneous examples associated with AfBPP

With respect to enzymes, clickable photoprobes for metalloaminopeptidases (MAPs) and GLO-1 have also been reported (Figure 16). In general, MAPs are exopeptidase enzymes that cleave a single N-terminal amino acid from a peptide substrate via hydrolysis. As a result of this catalysis, MAPs play very important roles in regulating metabolism and protein maturation in a number of organisms, including humans and bacteria, and these enzymes have been linked to multiple disease states such as hypertension and cancer. In particular, benzophenone-containing probe **95**, which is based on the *Actinoycetes* metabolite bestatin, was shown to function as an ABPP probe with specificity

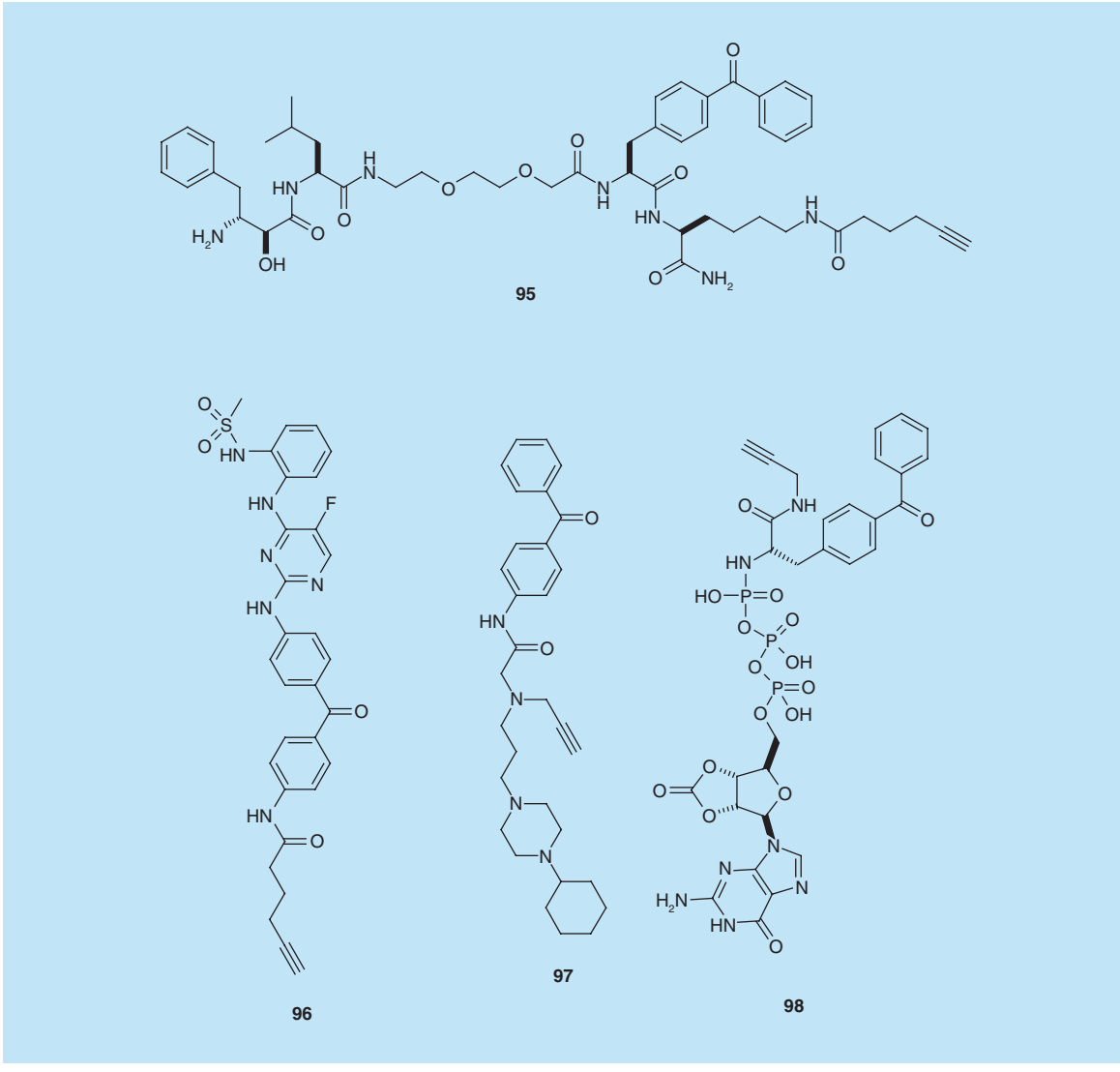

Figure 16. Miscellaneous clickable photoprobes associated with AfBPP.

for MAPs [66]. Specifically, the addition of an alkyne, a fluorophore or biotin to the lysine end of the compound did not change the activity-dependent labeling profile for the bestatin-based scaffold when subjected to a complex proteome. In contrast, GLO-1 is a highly expressed enzyme in cancer cells, which functions to quench 2-oxoaldehydes (e.g., methylglyoxal) as toxic metabolites. It has been previously shown that GLO-1 inhibitors can prevent proliferation of cancer cells and induce apoptosis, thus GLO-1 inhibitors represent potentially promising therapeutic entities in the area of cancer chemotherapy. In particular, 2,4-aminopyrimidine-based photoprobe L1-BPyne (**96**) was found to be a potent inhibitor of GLO-1 activity, wherein this compound can passively penetrate living cells and specifically label the enzyme *in situ* upon photoirradiation [67]. Current work associated with probe **96** is moving toward locating and mapping the binding site of this compound within GLO-1, principally as a means of gathering structural information toward developing future GLO-1 inhibitors with desirable properties.

With respect to receptor proteins, σ2 receptors are currently being targeted for diagnostic imaging purposes and pharmacological manipulation toward subduing cancer progression. Interestingly, an 18-kDa protein named σ2–18k was recently found to exhibit σ2-like photoaffinity labeling based on previous work employing radioactive photoprobes. However, the amino acid sequence and biological function of σ2–18k are not known. In order to provide tool compounds to study this protein, a series of clickable benzophenone photoprobes was generated, wherein probe **97** displayed 2 nM binding affinity for σ2 receptors and the greatest potency in terms of preventing photoaffinity labeling of σ2–18k by a previously reported radioactive photoprobe [68]. As a result, it is anticipated that probe **97** will aid in future chemical biology studies of σ2–18k to better understand the identity and function of this protein, particularly with the advantage of attaching a tag of choice after photoaffinity labeling versus radioisotope tags.

Finally, GTP-binding proteins are essential for cell trafficking, nucleotide metabolism, cell signaling, translation of mRNA into protein and cytoskeleton structure. With the goal of developing tools to study a variety of GTPases, clickable photoprobe GTP-BP-yne (**98**) was developed as an alternative GTP AfBPP probe to previously known radioactive GTP analogs [69]. In particular, benzophenone alkyne **98** proved useful in evaluating the purine nucleotide selectivities of target proteins found during AfBPP studies (i.e., several ATP-binding proteins were detected with probe **98**), and this work also led to the identification of a number of proteins previously unknown to bind to GTP, such as the ATPase enzyme BCS1L, where certain mutations of this enzyme leads to GRACILE syndrome.

Clickable photoprobes for characterizing the interactions of sterols or lipids with proteins

Sterol-related compound oleanic acid (**99**, Figure 17) possesses a wide variety of biological activities including anti-inflammation, anti-HIV, hepatoprotection, anti-hyperglycemia, anticancer and cardioprotection. However, the full complement of protein targets associated with the functional activities of oleanic acid has yet to be achieved. In order to obtain this incomplete information, clickable benzophenone probe **100** containing an azide chemical reporter, alongside a biotinylated analog also bearing a photoreactive benzophenone, were designed and synthesized based on previous SAR studies [70]. Unfortunately, clickable probe **100** showed fairly poor inhibition (IC_{50} = 115 μM, fivefold loss in activity) in an assay against RMGPa (i.e., a known target of oleanic acid) when compared with parent compound **99** (IC_{50} = 23 μM), whereas the biotinylated analog only displayed an approximately twofold loss in activity (IC_{50} = 41 μM). As a result of this difference in biological activity, the biotinylated benzophenone derivative was carried forward to photoaffinity labeling experiments instead of the clickable photoprobe.

Cholesterol is both an important precursor to numerous signaling compounds and a critical structural component of cell membranes. Cholesterol's biological effects and regulation stems from its specific interactions with numerous proteins. However, the full complement of cholesterol-binding proteins in mammalian cells has yet to be determined. Toward this end, a set of cholesterol-based clickable photoprobes (**101**) was generated in order to globally map the direct interactions of cholesterol with proteins in living cells [71]. This effort resulted in the identification of more than 250 proteins that bind to cholesterol, including previously unreported enzymes that regulate carbohydrates and glycerolipids, as well as those involved in protein degradation and glycosylation. This work is significant in that it points to key nodes in biochemical pathways that may link the control of metabolites, protein modification and protein localization to sterol concentrations.

Protein prenylation is a post-translational modification wherein an isoprenoid chain is conjugated to a protein. In particular, protein prenylation has been linked to many cellular processes associated with cancer and a host of metabolic diseases. As a result, this has led to the pursuit of prenyltransferase inhibitors as

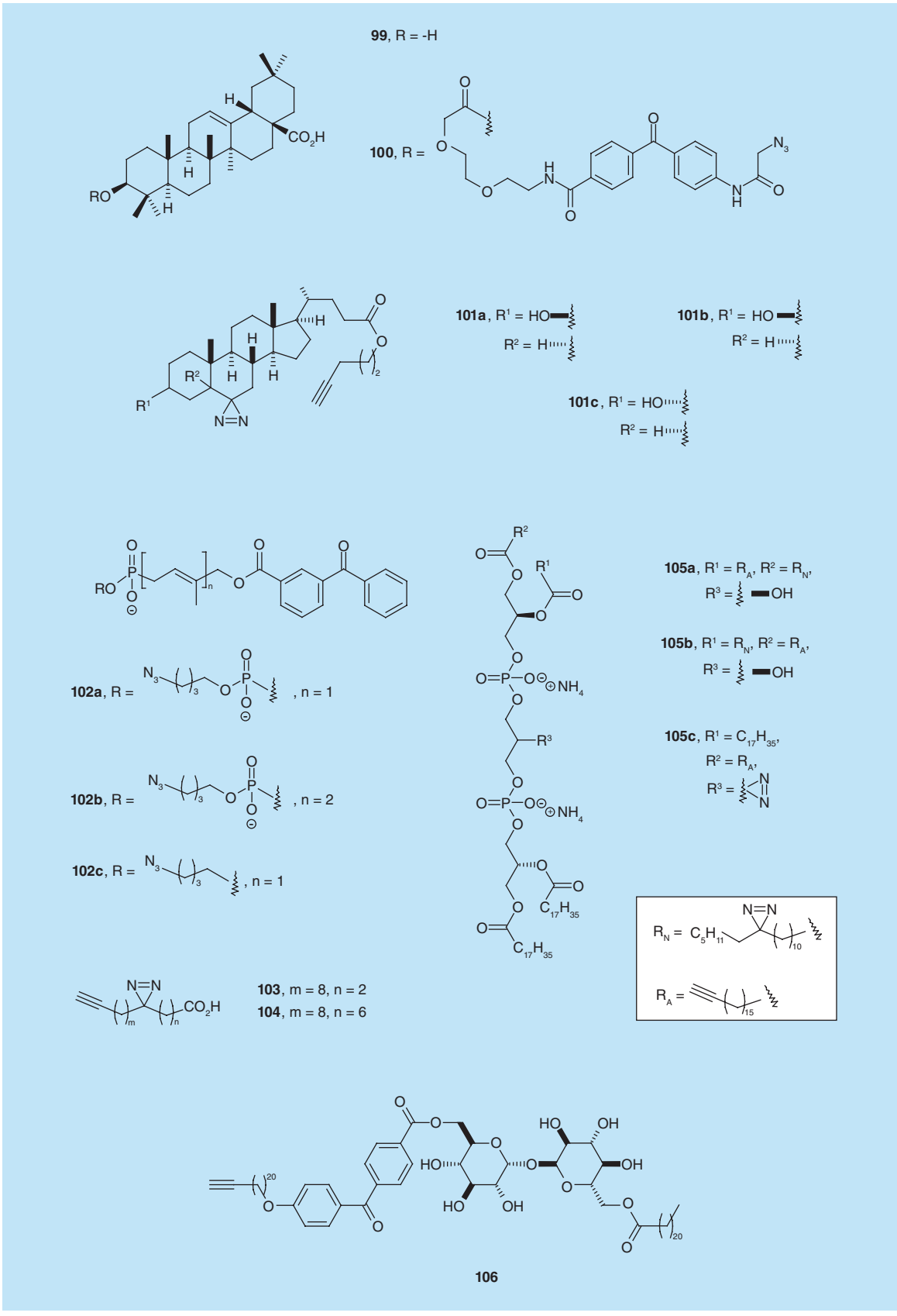

Figure 17. Clickable photoprobes for characterizing the interactions of sterols or lipids with proteins.

potential drug candidates for several disorders. Toward ultimately characterizing the detailed molecular interactions isoprenoids form with target proteins, clickable photoprobes **102** were prepared featuring an aliphatic azide chemical reporter and a photoreactive benzophenone unit, which is structurally similar to an isoprenoid chain [72]. In preliminary experiments, these probes were shown to be capable of covalently capturing proteins from a *Saccharomyces cerevisiae* cell lysate, namely via a sequence of photoaffinity labeling, bioorthogonal conjugation with an alkynyl biotin reporter, streptavidin enrichment and Western blot analysis.

With respect to clickable fatty acid-based photoprobes, cells were able to convert pacFA (**103**) into a variety of phospholipids that could covalently capture binding partners upon photoirradiation [73]. Subsequent bioorthogonal chemistry with a reporter of choice then allowed imaging and identification of the lipid-bound proteins *in situ*. Similarly, bifunctional fatty acid x-alk-16 (**104**) was capable of being metabolically incorporated into known *S*-palmitoylated proteins (e.g., IFITM3, H-Ras) in mammalian cells [74]. In particular upon in-cell photoactivation, x-alk-16 was able to induce covalent capture of IFITM3 protein oligomerization, as well as specific interactions of this potent antiviral protein with other membrane-bound proteins such as VAPA. As a result, x-alk-16 represents an important tool compound for characterizing additional *S*-palmitoylated membrane protein complexes in the future.

Cardiolipins are phospholipids containing four fatty acid acyl chains. In particular, these compounds are synthesized exclusively in the mitochondria and have been shown to participate in cellular apoptosis via specific interactions with cytochrome c as an apoptotic protein. Toward elucidating the details of the cardiolipin-cytochrome c complex at the molecular level, several cardiolipin-based diazirine photoprobes (**105**) were synthesized bearing a terminal alkyne clickable handle [75]. In turn, these compounds were shown to induce appropriate peroxidase activity of cytochrome c using liposomes reconstituted with the photoreactive cardiolipins. Currently, these probes are being subjected to photoaffinity labeling experimentation.

As a final example of a clickable lipid-based photoprobe, benzophenone **106** was designed, synthesized and evaluated as a trehalose diester AfBPP probe [76]. In particular, the most abundant glycolipids found in the cell wall of *M. tuberculosis* are trehalose dimycolates (TDMs), which are known to play a key role in the pathogenesis of this bacterial species. However, very little is known about the binding partners of TDMs in immune cells. Benzophenone photoprobe **106** was shown to demonstrate immune stimulating properties, principally by activating bone marrow derived macrophages to produce nitric oxide. Given this appropriate retention of biological activity, probe **106** is currently undergoing AfBPP studies as an effective TDM mimic.

Clickable photoprobes for determining binding sites within target proteins

Alternative to macrolevel analysis of photoprobe labeled target complexes (i.e., **4**; Figure 1), photoaffinity label-

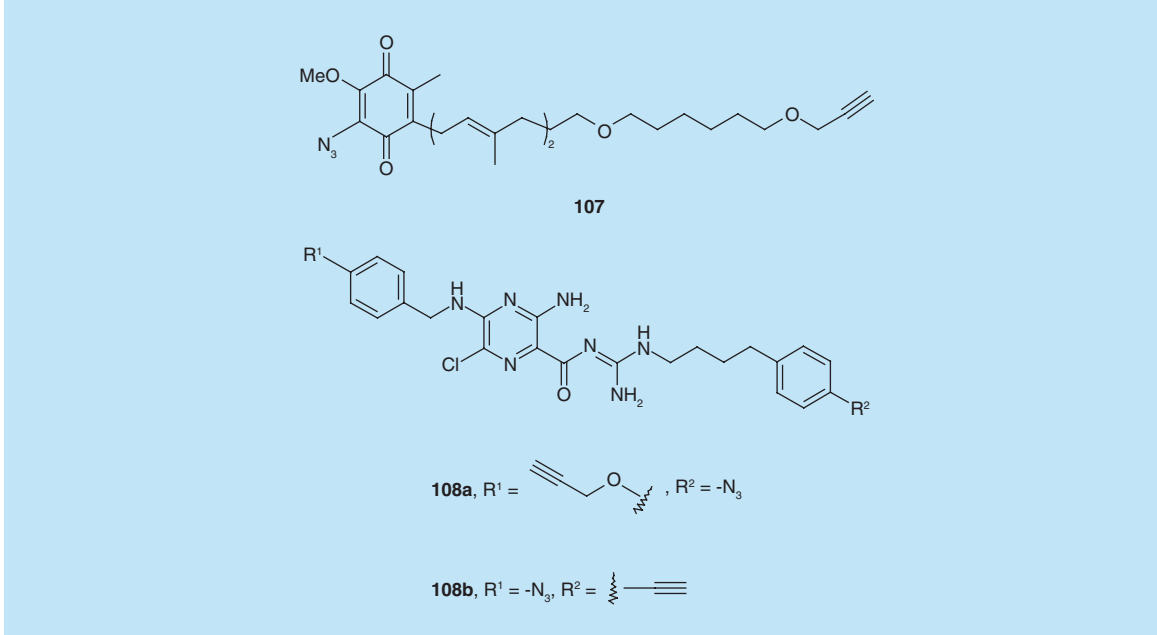

Figure 18. Clickable photoprobes for determining binding sites within target proteins.

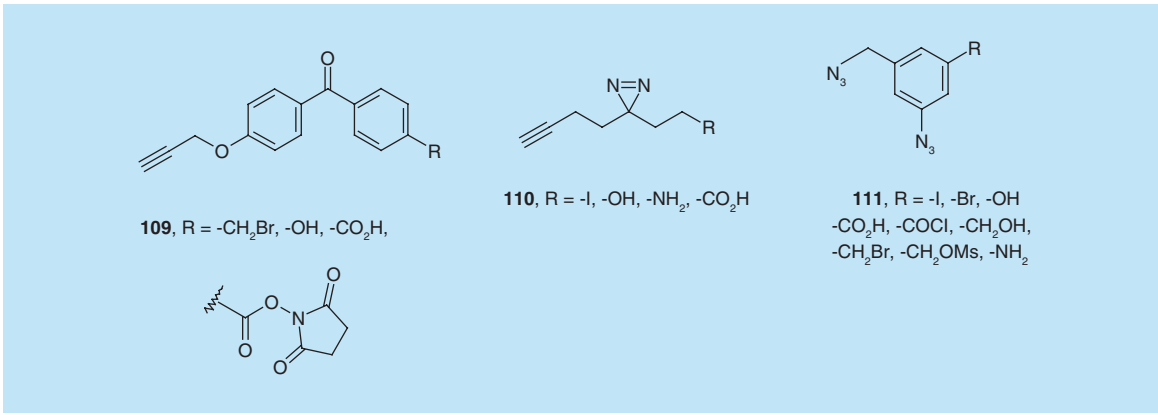

Figure 19. Minimalist building blocks containing a photoreactive group and a click chemistry handle.

ing can also be used to locate ligand-binding sites within target proteins (i.e., microlevel analysis). For example, tandem photoaffinity labeling-bioorthogonal conjugation was performed using aryl azide based ubiquinone probe **107** (Figure 18) in order to elucidate the binding site of the quinone group in recombinant *Schizosaccharomyces pombe* Coq10, an enzyme involved in ubiquinone biosynthesis [77]. Specifically, the quinone moiety of this compound was found to bind to the N-terminal region of Phe39-Lys45 in *S. pombe* Coq10 upon detailed proteomic analysis. In short, photoprobe **107** is expected to aid in further delineation of the physiological role of Coq10, which currently remains elusive.

As an additional example of binding site determination via tandem photoaffinity labeling-bioorthogonal conjugation, benzamil photoprobes **108** were used to confirm that amilorides bind to the quinone-binding pocket within bovine mitochondrial complex I, an enzyme involved in ubiquinone reduction [78]. Specifically, these aryl azide based probes were found not to label any of the antiporter-like subunits of the protein complex. Instead, probes **108** were shown to specifically label the accessory subunit B14.5a and the N-terminal region Thr25-Glu115 of a 49-kDa core subunit. In short, these proteomic results confirm that amilorides inhibit the activity of bovine mitochondrial complex I by occupying the quinone-binding pocket of this complex, not by blocking the movement of protons through antiporter-like subunits.

Conclusion & future perspective

Photoaffinity labeling was first introduced by Westheimer's group in the 1960's [79]. Since that time, photoaffinity labeling has become a powerful method to covalently capture the protein targets of small molecules. In particular, photoaffinity labeling has seen a resurgence in recent years, in part due to the advancement of proteomic methods for the identification of labeled targets. Additionally, the development of bioorthogonal/click chemistry conjugation reactions has allowed for the design of clickable photoprobes with minimal modifications to the parent lead molecule. This has expanded the versatility of photoaffinity labeling because, with the design of a single clickable photoprobe, various reporter groups can be attached after photoaffinity labeling in order to visualize or enrich/identify labeled proteins. Furthermore, clickable photoprobes can be used in live cells because a simple alkyne analog is more likely to penetrate cells than the corresponding biotinylated photoprobe. Additionally, clickable photoprobes are more likely to distribute uniformly throughout the cell compared with many fluorophore-linked photoprobes.

In the absence of binding site information, it is prudent to not limit photoprobe design to one type of photoreactive group, but rather include at least one carbene- or nitrene-based photoprobe, plus one benzophenone-based photoprobe, in order to maximize chances for success. If the photoreactive group within the photoprobe is bound within the ligand-binding site, it is likely that carbene and nitrene reactive species generated upon photoirradiation will be preferred, since these reactive species are smaller in size, more likely to be sterically tolerated, and are more chemically reactive compared with ketyl radical reactive species generated upon benzophenone photoirradiation. In contrast, if the photoreactive group within the photoprobe is protruding out of the ligand-binding site and is solvent exposed, benzophenone ketyl radical reactive species will be preferred, principally because if this reactive species gets quenched by solvent, it can potentially be regenerated under continued UV irradiation until a productive probe-protein crosslink is formed. In contrast, nitrenes and carbenes are more reactive and are more likely to be completely quenched if solvent exposed, wherein once these reactive species are quenched by solvent, they cannot be regenerated upon continued photoirradiation. Another practical con-

sideration is that the benzophenone functional group has additional stereoelectronic constraints, wherein disruption of its conjugation upon binding can lead to less efficient formation of the ketyl diradical reactive species. Additionally, it has been reported that the benzophenone photoreactive group preferentially crosslinks to methionine, which can bias crosslinking to proteins with readily accessible methionines [80].

Another important consideration is the placement and type of clickable tag employed within the design of the photoprobe. A terminal alkyne is most often incorporated within the photoprobe structure, whereas the complementary aliphatic azide click chemistry handle is typically placed on the reporter (i.e., TAMRA-azide or biotin-azide), although the reverse arrangement is possible. The reason for these preferences is that more nonspecific background labeling tends to be observed with alkyne-linked fluorophores. More recently, minimalist clickable benzophenone, diazirine and aryl azide building blocks have been designed that incorporate both a photoreactive group and a click chemistry handle in a single reagent (Figure 19). Select examples of these minimalist moieties include propargyl ether benzophenones **109** [20,43,59,60,81,82], alkynyl alkyl diazirines **110** [44,45,65] and diazides **111** [2,37,39,61,62,83,84], which can be used to conveniently prepare a variety of clickable photoprobes in a library approach (e.g., [85]).

In summary, clickable photoprobes have greatly expanded the utility of photoaffinity labeling for a number of different applications, including target identification, confirmation of target engagement and selectivity profiling. Looking forward, we expect tandem photoaffinity labeling-bioorthogonal conjugation will continue to play a prominent role in target identification studies, especially given the recent return to phenotypic screening as a strategy to discover first-in-class therapeutics [86]. In particular, clickable photoprobes are well-suited for capturing small molecule target interactions in a live cell setting. Additionally, these probes are capable of detecting and/or enriching membrane protein targets, principally because the probe–target complex is covalent in nature, and it can be solubilized without losing the probe-interacting targets for downstream analysis.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Executive summary

- The rational design of photoprobes appears to be moving away from the direct incorporation of radioisotopes, biotin, fluorophores and epitope tags (e.g., FLAG peptides) and instead is moving toward the routine incorporation of bioorthogonal/click chemistry handles as latent chemical reporters.
- Tandem photoaffinity labeling-bioorthogonal conjugation has a number of powerful applications including target identification of hit compounds originating from phenotypic screens, affinity-based protein profiling, characterization of lipid–protein interactions and binding site determination.
- Clickable photoprobes allow photoaffinity labeling not only in cell lysates, but also live cells as well, the latter giving a more accurate representation of ligand–target interactions that naturally occur in a physiological setting.

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Review
Special Focus Issue: Chemical Biology

Future Medicinal Chemistry

Chemical genetics and regeneration

Regeneration involves interactions between multiple signaling pathways acting in a spatially and temporally complex manner. As signaling pathways are highly conserved, understanding how regeneration is controlled in animal models exhibiting robust regenerative capacities should aid efforts to stimulate repair in humans. One way to discover molecular regulators of regeneration is to alter gene/protein function and quantify effect(s) on the regenerative process: dedifferentiation/reprograming, stem/progenitor proliferation, migration/remodeling, progenitor cell differentiation and resolution. A powerful approach for applying this strategy to regenerative biology is chemical genetics, the use of small-molecule modulators of specific targets or signaling pathways. Here, we review advances that have been made using chemical genetics for hypothesis-focused and discovery-driven studies aimed at furthering understanding of how regeneration is controlled.

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Regenerative biology explores how lost body parts, appendages, tissues or cells are replaced. Interest in regenerative processes extends to Aristotle's time; yet despite establishing experimental biology as a disciplined practice [1], regenerative biology has largely been limited to descriptive dissertations throughout much of its history. Today, with new genetic and imaging methodologies applicable to a wide variety of regenerative model species, the field abounds with fresh insights into the cellular and molecular mechanisms controlling regeneration. In addition, the advent of embryonic and induced pluripotent stem cells (ESC and iPSC, respectively) has spawned a new field, regenerative medicine, emphasizing the development of therapeutic strategies for reversing the course of degenerative diseases in humans.

Currently, there are two main approaches applied to regenerative therapeutics: first, transplantation of cells derived from differentiated stem cell cultures and; second, stimulation of the regenerative potential of endogenous stem cells to repair damaged tissues or replace lost cells. Within the field of chemical biology, testing and screening small-molecule modulators of molecular targets and signaling pathways have the potential to bridge these two approaches by revealing common mechanisms for controlling stem cells; in other words, pathways for regulating reprogramming/dedifferentiation, proliferation and differentiation of stem cell cultures and within the context discrete stem cell niches *in vivo*. Due to the comparative ease of *in vitro* testing, the vast majority of insights into stem cell biology using small molecules have come from efforts to increase reprogramming efficiency, maintain pluripotency or direct differentiation of ESC and iPSC cultures. Accordingly, many exceptional reviews have covered these topics [2–6] as well as concomitant advances in small-molecule chemistry [7,8]. In this review, we focus on contributions that chemical biology has made to classical regenerative biology, within the context of whole-organism screening in model species exhibiting robust reparative mechanisms. We will cover hypothesis-driven studies ('reverse' chemical genetics) and discovery-oriented screens ('forward' chemical genetics), emphasizing common paradigms and representative

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Key terms

Regeneration: The process of replacing lost tissues/cells.

Dedifferentiation: The process by which somatic cells can convert to a stem cell-like state, characterized by the upregulation of genes associated with an earlier stem/progenitor state and serve as a source of new cells during regeneration.

Differentiation: The process of cell fate acquisition in which a cell exits the cell cycle and expresses genes delineating a specific lineage and/or cell type.

Chemical genetics: The use of the chemical modulators to investigate the role of molecules and molecular signaling pathways in biological processes of interest.

studies for each strategy (Figure 1). In addition, we will discuss how the scale of injury likely affects the nature of the regenerative process and impacts model amenability to large-scale assay platforms. Finally, we will integrate how insights from this work could aid the development of regenerative therapeutics.

Chemicals & biology

The use of chemicals to modulate universal biological processes – such as mitosis (colchicine), transcription (actinomycin D) and translation (cyclohexemide) – has a long and productive history. Similarly, application of chemical modulators of more discrete molecular targets has been a common practice in multiple biological disciplines for decades (e.g., neurotransmitter inhibitors). However, systematic use of small molecules to probe gene function or to pursue large-scale drug discovery, in other words, 'pharmacological' [9] or 'chemical' genetics [10] necessitated the development of combinatorial chemistry. The ability to synthesize large libraries of chemical variants availed targeting of individual gene products with high specificity. Interestingly, an initial reductionist emphasis (one drug, one target) has recently evolved to embrace the reality of polypharmacology (one drug, multiple targets) as both a complication to overcome [11] and a potential advantage to leverage [12,13] in high-throughput screening (HTS).

Compared with genetic manipulations, chemical modulators provide several significant advantages: first, temporal control – the ability to limit compound exposures to specific stages or reverse effects upon 'washout'; second, graded responses – titrations can be used to elicit dose-dependent effects, inducing phenotypes akin to an allelic series of genetic mutants and third, refractory to redundancy or genetic compensation – modulators acting on homologs, common downstream signaling molecules, or even entire gene families can circumvent issues arising with single gene manipulations [14]. When applied as a platform for discovery, large-scale chemical screens can reveal new insights into almost any biological process of interest. However, one of the confounding factors associated with chemical-based approaches is nonspecificity due to: first, effects on multiple members of a protein family or, second, modulations of pathways other than the intended target. The first issue can actually be viewed as a strength, circumventing genetic compensation/redundancy issues by using a pan-family modulator to target an entire class of proteins. As a test for specificity of observed effects, both issues may be addressed by either testing multiple modulators of the implicated protein/pathway or using dose-response strategies and titrating to a level that promotes specificity of binding. Nevertheless, questions of specificity may cloud interpretations of chemical biology assays and efforts to allay this concern should be pursued vigorously when this methodology is a central component of a study.

As mentioned above, the vast majority of chemical biology screens have utilized cell culture systems. This has the advantages of straightforward treatment regimens and reduced toxicity compared with *in vivo* systems. However, despite their simplified nature, reductionist approaches have not been particularly successful for drug discovery [15]. Conversely, serendipitous discovery of compounds eliciting specific phenotypic effects – in other words, phenotypic screening has played a long and storied role in drug development [16]. To adapt this approach to chemical genetics, several groups have begun to perform large-scale drug screens directly in living animal models [17–22] (see Table 1 for a list of the research discussed below and additional studies). Here, we focus on recent applications of chemical genetics to regenerative biology spanning organismal, appendage, organ and cellular replacement paradigms.

Regenerative biology & chemical genetics

It is often said that 'regeneration recapitulates development'. Indeed, regenerative paradigms involve developmental signaling pathways regulating the proliferation, differentiation and patterning of stem cells and their progeny [23]. Classical genetic approaches to studying regeneration are somewhat stymied therefore by the need to implement conditional approaches, such as temperature-sensitive screens, to circumvent lethal or altered morphology phenotypes. Thus, a key advantage of applying chemical versus classical genetics to regenerative biology is that it more readily facilitates temporal dissection of the roles played by developmental signaling pathways. However, the particular cellular mechanisms used to replace lost tissue can show remarkable context specificity, both across species and between different organs within the same species, some of which are not typically associated with development (Table 2). Moreover, environmental factors that shaped developmental

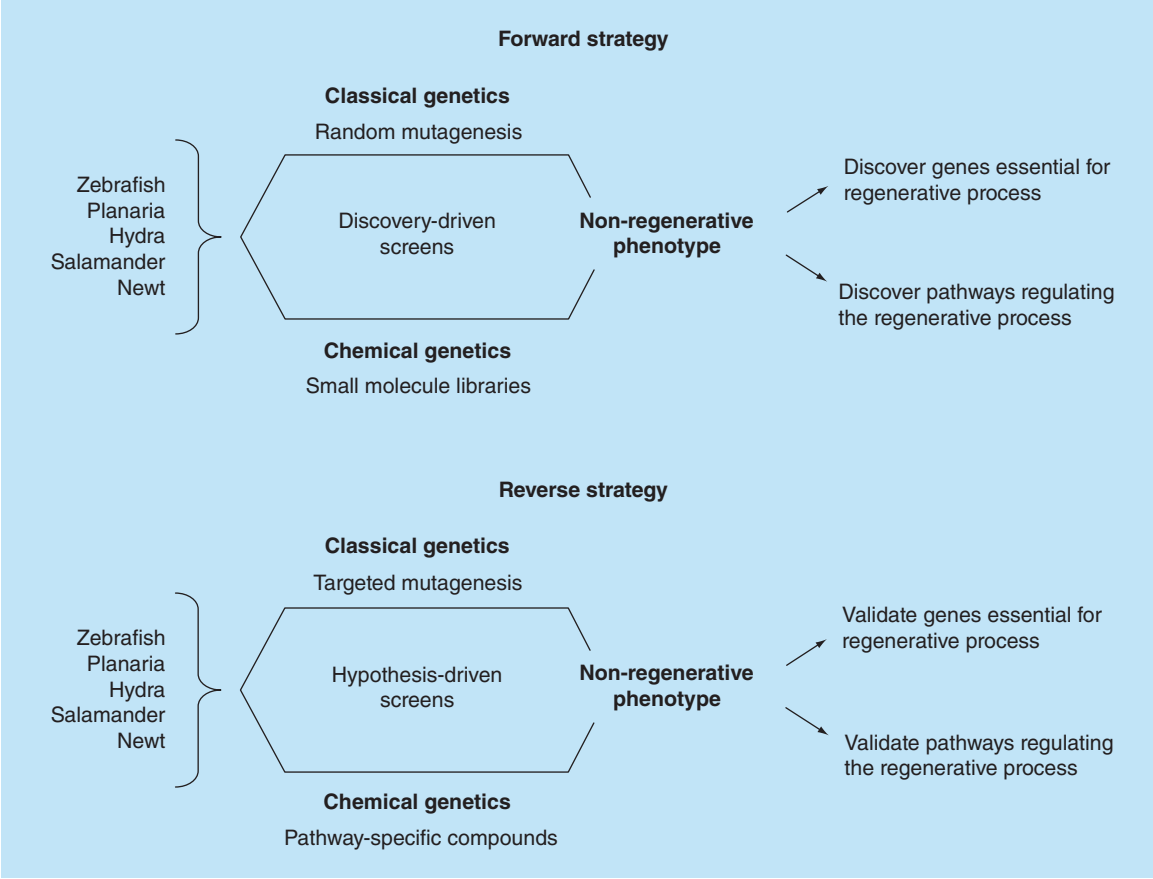

Figure 1. The forward strategy is a discovery-driven screening approach whereby genes/chemicals are randomly tested for effects on a phenotype of interest – the emphasis is on disrupting the phenotype first, then determining which genes/pathways are involved. The reverse strategy is a hypothesis-driven approach that uses prior knowledge to select a given gene/pathway to investigate regarding a regenerative paradigm of interest – the emphasis is on disrupting a gene/pathway first (either by knocking it down or inhibiting function using small-molecule inhibitors) and determining phenotypic effects secondarily.

events are unlikely to be maintained at more mature stages. Thus, it is important to keep in mind that much remains to be discovered; in other words, cellular and molecular mechanisms at play during regenerative processes may be discrete from developmental pathways. A broader understanding of the combinatorial interactions among components of discrete stem cell niches and between implicated signaling pathways should help to define ways to stimulate endogenous repair mechanisms in humans. In the following sections, we will highlight studies that have applied chemical genetics in regenerative model species to reveal molecular factors that impinge upon regenerative processes. We begin by discussing studies that have used hypothesis-driven reverse chemical genetics to explore molecular mechanisms controlling regeneration.

Reverse chemical genetics

Organismal regeneration: planaria

A handful of remarkable species are able to regenerate completely after being transected. For instance, planaria can completely regenerate from small fragments containing stem cells known as neoblasts [24]. Moreover, transplanting a single neoblast cell, the clonogenic neoblast, can rescue a lethally irradiated host [25]. Following injury (e.g., bisection), neoblasts respond by proliferating and migrating toward the wound site, giving rise to progeny that form the regenerative blastema [26], a group of dedifferentiating mesenchymal cells that aggregate beneath the injury site following wound healing. Surviving cells also undergo remodeling to integrate with the newly generated cells. The molecular signaling events regulating this process have yet to be fully characterized. Early key mechanistic hypotheses were developed in planaria by applying anesthetics and inhibitors of respiration, mitosis or protein synthesis, demonstrating the value of chemical biology to regenerative paradigms [27]. More recently, studies using long-term RNAi have implicated classical developmental signaling pathways (e.g., BMP, Hedgehog, Wnt) in regulating patterning during regeneration and numerous genes in modulating neoblast proliferation [28–30].

Table 1. Forward genetic studies.

Tissue	*In vivo* or *in vitro*	Model system	Number of compound	Identified targets	Ref.
Bone	*In vitro*	Immortalized murine osteoblast cell line	30,000	Statins	[127]
	In vitro	Myoblast with BMP2 treatment	5405	Rapamycin and FK-506	[128]
	In vitro	Preosteoblastic MC3T3E1 cells by expressing GFP	2500	Glabrisoflavone (GI)	[156]
	In vitro	Mesenchymal stem cells	1280	Raf–MEK–ERK pathway targeting osteogenic factors	[130]
Fin	*In vivo*	Wild-type larval zebrafish	2000	Glucocorticoids	[125]
	In vivo	Transgenic larval zebrafish	520	Imidazoline receptor antagonist	[126]
Heart	*In vitro*	Pluripotent mouse stem cell line (P19CL6)	147,000	Sulfonylhydrazones	[132]
	In vitro	Mouse embryonic stem cell line	550	Wnt inhibitor	[134]
	In vitro	Mouse embryonic stem cell-derived cardiomyocyte	280	Inhibitors of glycogen synthase kinase-3, p38 mitogen-activated protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II and activators of extracellular signal-regulated kinase	[133]
Hair cell	*In vivo*	Multiple larval zebrafish transgenic line	1680	Topoisomerase activity and cell cycle	[135]
	In vivo	Wild-type and multiple larval zebrafish transgenic lines	470	Fucoidan	[136]
Pancreas	*In vivo*	Zebrafish transgenic line	7186	Adenosine pathway	[145]
	In vivo	Zebrafish transgenic line	3131	Retinoic acid and GTP	[150]
	In vivo	Zebrafish transgenic line	833	Retinoic acid, serotonergic signaling, glucocorticoids	[151]
	In vivo	Zebrafish transgenic line	Over 500,000	NF-κB pathway	[152]
	In vitro	Primary rodent and porcine islet β cells	850	Adenosine	[148]
Muscle	*In vivo*	Zebrafish sapje and sapje-like fish	1120	Aminophylline	[143]
	In vivo	Zebrafish sapje	640	Fluoxetine	[142]

Pharmacological inhibitors of candidate signaling pathways have been used to complement RNAi studies. For instance, Tasaki *et al.* used the MAPK/ERK kinase (MEK) inhibitor U0126 to demonstrate that reductions in ERK signaling maintained blastemal cells in a proliferative state, thus blocking differentiation. This effect could be rescued by knocking down expression of a MAPK phosphatase (*mkpA*) with RNAi [31], presumably by enhancing residual ERK activity. The same group has shown that ERK activity specifically promotes 'head' differentiation during regeneration, acting in opposition to posterior Wnt signals [32]. MEK inhibition also demonstrated that crosstalk between ERK and Wnt signaling is necessary for regeneration of the pharyngeal apparatus – in other words, the area between the head and tail regions. Interestingly, using a different MEK inhibitor (PD 98059), Ermakov *et al.*, found that proliferation outside the blastema is actually reduced [33], but saw similar disruptions in head differentiation.

Furthermore, chemical genetics experiments in planaria have revealed a role for gap junctions during regeneration. Gap junction (GJ) proteins are specialized channel proteins located in the plasma membrane and essential for cell–cell communication. In planaria, the innexin family of GJ proteins (invertebrate

homologs of connexins) consists of at least a dozen members expressed in semioverlapping domains [34]. Thus, to completely disrupt GJ function during regeneration would require the coordinated action of multiple RNAi oligos. Alternatively, a single pan-innexin chemical inhibitor that disrupts the entire innexin family, such as heptanol, can suffice to block all GJ communication. Nogi and Levin applied this method to explore the role of GJ communication in planarian regeneration [35]. Transient exposures to heptanol during the first 2 days of regeneration following amputation resulted in 'anteriorization' of the posterior blastema, characterized by a lack of tail regeneration or the appearance of second head in the posterior segment. This study not only discovered a critical role for innexins in anterior–posterior (AP) polarization during regeneration but also demonstrates an important advantage of chemical biology, and one that is typically seen as a complication to be surmounted: nonspecificity. Here, a single chemical reagent was used to disrupt an entire protein family to achieve the desired effect on signaling, an outcome that would have been difficult to achieve with genetic manipulations due to redundancy, compensation and/or combinatorial applications of gene knockdown toolsets. This study was followed up by Oviedo *et al.* to explore how anteriorized regenerative structures reacted to subsequent amputations [36]. To induce anteriorization, they used timed exposures to an optimized dose of another GJ inhibitor, octanol, which blocked only a subset GJ types but allowed normal neoblast function. When ectopic heads were reamputated up to 6 weeks later, thus in the absence of any molecular manipulation, regenerated structures retained the respecified head morphology. This study thus demonstrated that a brief disruption of GJ communication is sufficient to induce persistent physiological alterations in patterning of regenerated structures without alteration of the genome, a remarkable observation with far-reaching implications.

Similar studies have examined the role of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMP) in extracellular matrix (ECM) remodeling during regeneration [37]. Balestini *et al.* found that the natural alkaloid berberine could disrupt planarian head regeneration, resulting in malformation of visual system, while tail regeneration proceeded normally [34]. BrdU-labeling and anti-pH3 staining indicated the berberine effects were not through alterations in cell proliferation, instead berberine significantly reduced expression of *Dj-mmp1*, *Dj-mt-mmpa*, *Dj-ast4* and *Dj-timp*. This finding provided direct evidence that MMPs and TIMPs are important to the regenerative process in planaria.

In addition to enabling studies on biochemical signaling, chemical genetics also facilitates investigation of biophysical signaling events that coordinate regeneration. A recent study by Beane *et al.* explored the effect of ionic gradients on axial polarity during planarian regeneration using ion transport inhibitors to modulate membrane voltage [38]. Exposure to the compound SCH-28080 specifically inhibited H,K-ATPases (ion transporters responsible for depolarizing the anterior blastema during regeneration), induced hyperpolarization and resulted in a headless regener-

Table 2. List of animal models with regenerative ability.

Animal model	Regenerative capacity	Adaptable for HTS	Ref.
Invertebrates			
Hydra	All tissues	Yes	[157]
Planaria	All tissues	Yes	[158]
Drosophila	Imaginal discs	Yes	[159]
Cockroach	Leg	Yes	[160]
Vertebrates			
Newts	Limbs, tail, heart, lens, spinal cord, brain, jaw, retina, hair cells of the inner ear	No	[161]
Frogs	Premetamorphic limbs, tail, retina, lens, hair cells of the inner ear	No	[162]
Zebrafish	Fins, tail, heart, liver, spinal cord, hair cells of inner ear, lateral line	Yes	[163]
Chicks	Hair cell of the inner ear	No	[164]
Mice	Liver, digit tips	No	[165,166]
Deer	Antler	No	[167]

HTS: High-throughput screening.

ate. The researchers hypothesized that the effects of membrane voltage manipulations might be mediated via changes in calcium flux. Indeed, inhibition of L-type voltage-gated calcium channels (using nicardipine) also resulted in reductions in head regeneration. Conversely, modulation of chloride flux (using ivermectin to keep glutamate-gated chloride channels open), or activating voltage-gated calcium channels (using praziquantel), resulted in a two-headed phenotype [39]. In addition, H,K-ATPase was shown to regulate left–right asymmetrical patterning [40] and apoptosis, thus disrupting remodeling in the head [41]. Chemical modulators leading to headless or two-headed phenotypes induced corresponding changes in the expression of anterior/posterior transcriptional factors in the blastema. Collectively, the data suggest that membrane voltage reiteratively comes into play during planarian regeneration to regulate several key steps including: specifying polarity, apoptosis, remodeling, proliferation and transcription.

Epimorphic regeneration: appendage replacement

Appendage regeneration (e.g., limbs, fins, digits, etc.) proceeds through fundamental stages of wound healing, blastema formation and patterning [42]. Lineage-tracing studies have generated insights into blastema formation. Interestingly, the blastema retains spatial memory, establishing a proximal/distal axis early on and maintaining it throughout the regenerative process [42,43]. Multiple studies have used chemical modulators to reveal key developmental signaling pathways, such as Wnt and Fgf [44], that are important for blastema formation and proximal/distal axis maintenance.

The Levin group has utilized chemical genetics to explore the role of ion currents in tail regeneration in *Xenopus laevis* utilizing an inhibitor of voltage-gated sodium channels (called tricaine or MS222) [45]. Sodium channel blockade inhibited tail regeneration, revealing that sodium ion influx was critical for a successful regenerative response. Their study further demonstrated that sodium influx was important during initial stages of repair as exposure in the first few hours after injury was sufficient to prevent regeneration. A screen for the presence or absence of known signaling pathways markers further demonstrated that inhibition of sodium ion influx impaired regeneration by modulating Notch and Msx1 induction, thus correlating biophysical signaling with biochemical signaling during appendage replacement. Moreover, transient activation of sodium ion influx during the wound-healing stage using the chemical activator monensin resulted in enhanced regeneration during a refractory period where regeneration does not normally occur. This study demonstrated that stage-specific modulations are important for identifying enhancers of regeneration as well as to decipher sequences of signaling events during regenerative processes. It also highlights a key advantage to using chemical as opposed to genetic modulators: improved temporal control over pathway manipulations.

Another attractive platform to study regeneration is the zebrafish caudal fin. In 1995, Johnson and Weston described an ENU-directed genetic screen for mutations that inhibit adult tail fin regeneration [46], demonstrating the advantages of applying forward screening strategies to regeneration in zebrafish (see below for further discussion). Since then, multiple studies have utilized transgenic/mutant fish and chemical modulators to reveal specific roles for signaling pathways such as Wnt [47], FGF [48] and Notch [49].

Both adult and larval zebrafish are capable of regenerating fins. Many markers for each phase of regeneration, such as *dlx5* for wound epithelium and *msxE* for blastema, are also conserved between different appendage regeneration models [50,51]. In addition, *raldh2*-mediated retinoic acid (RA) signaling regulates several appendage/tissue regeneration paradigms [52,53]. A chemical genetics study used the timing of *raldh2* expression in the blastema of the regenerating larval fin to investigate a panel of molecular regulators of appendage regeneration. However, not only does chemical genetics identify signaling pathways involved during regeneration but it can also reveal the hierarchy of signaling interactions across pathways. For example, inhibitors of FGF (SU5402) and ERK (UO126) block larval fin regeneration. However, RA coexposure rescued the effects of these pharmacological inhibitors, suggesting that RA signaling acts downstream of FGF and ERK during regeneration [54].

A more recent chemical genetics study sought to reveal additional signaling pathways involved in cell proliferation and migration responses postinjury [55]. Inhibitors of different oncogenic pathways were tested in the larval fin regeneration model by looking for any change in the proliferative response following amputation. Interestingly, p38 and MEK1 inhibition resulted in an increase in proliferation while PI3K and ErbB inhibition caused a decrease in proliferation within the wound epithelium and blastema; these effects were additive when fish were treated concurrently with inhibitors to both pathways. In addition, ErbB/PI3K inhibition could also abolish migration of cells into the blastemal region. Finally, ErbB impairment arrested regeneration when fish were exposed at later stages, suggesting the ErbB is required for proliferation throughout of the regenerative process. Together these

data imply that ErbB and PI3K interact functionally to impact cell proliferation and cell migration during regeneration. These and a host of similar studies have demonstrated the usefulness of chemical genetics for revealing new insights into signaling pathways involved in epimorphic regeneration.

Tissue regeneration

Appendage regeneration involves the replacement of complex multitissue structures. It is therefore reasonable to assume that epimorphic regeneration largely follows a developmental program. Conversely, tissue regeneration is restricted to a more discrete landscape, sometimes involving a single stem cell niche. Accordingly, it is less clear to what extent tissue regeneration 'recapitulates development' or whether pathways specific to the regenerative process are called into play as well. Below we will examine how chemical genetics has determined some of the key signaling mechanisms involved in two important tissue regeneration models.

Bone

Bone loss is a common health problem incurred as a result of injuries, aging and disease. Human bones can regenerate after injury following a well-characterized healing and remodeling process [56,57]. However, when large quantities of bone mass are lost, our regenerative capacities can be outstripped. Better understandings of the mechanisms regulating bone regeneration are thus needed to facilitate more effective bone repair.

It is known that crosstalk between osteoblast and osteoclast cells maintains bone homeostasis [58]. Many transcription factors (e.g., *sox9*, *runx2*, *osx*, *atf4*, *ap1*) and signaling pathways (Hh, Wnt, Notch, BMP, FGF) are critical for osteoblast differentiation and thus may be useful therapeutically [59–61]. For instance, recombinant BMP proteins have been used to treat bone disease [62]. However, recombinant proteins have multiple limitations, which restrict their application [63]. Accordingly, small molecules are being used to target pathways regulating bone regeneration, such as osteoblast differentiation [63,64].

Osteogenesis in zebrafish scales and mammalian bone utilizes similar signaling mechanisms [65]. Based on previous studies implicating Wnt/Sp7 interactions in osteoblast differentiation [66], De Vrieze *et al.* screened a small library of Wnt modulators in an *ex vivo* zebrafish scale culture model [67]. For this, they developed a transgenic line in which luciferase was driven by the Sp7/osterix promoter, enabling screens for factors promoting osteoblast differentiation. In their proof-of-principle study, they accurately predicted the effects of 70% of characterized Wnt modulators and identified riluzole, genistein and niclosamide as having strong osteogenic activity [67]. It will be extremely interesting to learn how well these findings 'translate' to mammalian model systems as this particular system is well-suited to large-scale forward discovery screens (see below).

Heart

Amphibians [68], fish [69–71] and neonatal mice [72] have the ability to regenerate heart tissue after injury; however, human cardiomyocytes have an extremely limited capacity to regenerate following injury or disease [73]. Thus, finding ways to enhance this ability in humans has garnered a great deal of attention. Importantly, zebrafish heart regeneration also involves proliferation of cardiomyocytes postinjury, thus providing a model to reveal pertinent signaling pathways. Using a fluorescent ubiquitination-based cell cycle indicator (FUCCI) system, Choi *et al.* identified several compounds that modulate cardiomyocyte proliferation in zebrafish embryos. In particular, they found that the Hh, Igf and Tgfβ pathways all stimulate cardiomyocyte proliferation during development [74]. They further demonstrated that these compounds also have similar effects on cardiomyocyte proliferation during heart regeneration. Similarly, Huang *et al.* and Chablais and Jazwinska found roles for Igf and Tgfβ, respectively, during heart regeneration.

Using NVP-AEW-541, a pharmacological inhibitor of the Igf1 receptor, Huang *et al.* demonstrated that inhibiting Igf signaling impairs cardiac regeneration by inhibiting proliferation of cardiomyocytes [75]. Specifically, Igf signaling appears to play a critical role in regulating proliferation of a subpopulation of gata4:GFP-labeled subepicardial cardiomyocytes postinjury. This subpopulation migrates to the wound site and proliferates, and is believed to be a primary source for new cardiomyocytes during heart regeneration [76]. Thus, Igf signaling is implicated in controlling the regenerative potential of the heart by modulation of a subset of cells that act as cardiomyocyte stem cells.

In addition, the Tgfβ pathway regulates three discrete aspects of heart regeneration. Using the compound SB431542 to block signaling from Tgfβ-type I receptors, Chablais and Jazwinska found cardiac regeneration was disrupted following cryoinjury. To dissect the function of Tgfβ signaling at different stages of the reparative and regenerative processes, they limited exposure to SB431542 to three discrete windows of time [77]. This strategy demonstrated that Tgfβ signaling was essential: initial scar formation – revealing this temporary collagenous tissue at the early-stage postdamage (14 dpci: 14 days postcryoinjury). Besides for collagen, it was also required for the deposition of other ECM, such as fibronectin and tenascins, which

are essential for ECM remodeling. A short-period Tgfβ inhibitor exposure after a 7-day recovery showed a significant reduction of proliferating myocytes in the boundary of the injury, which indicated Tgfβ had stimulating role in cardiomyocyte proliferation.

While individual signaling pathways may have discrete effects on regenerative processes, it is important to understand how multiple pathways integrate following injury as well. Based on previous studies reporting that either FGF1 treatments or p38MAK inhibition can decrease cardiomyocyte apoptosis in ischemic heart disease [78,79], Engel *et al.* investigated the result of combining p38MAPK inhibition with exogenous FGF1 [80]. Their study revealed that in an acute myocardial injury, combining FGF1 and p38MAPK inhibitor treatments increased cardiomyocyte proliferation as well as improved and extended cardiac function compared with administration of FGF1 or p38MAPK inhibitors alone.

Cellular regeneration

While tissue and appendage regeneration paradigms have clear clinical significance, the majority of diseases associated with the promise of stem cell biology are degenerative or autoimmune disorders involving the progressive loss of specific cell types (e.g., Parkinson's disease). The study of cellular regeneration, as a distinct regenerative biology paradigm, will therefore be important for defining cell-specific stem cell niches and for discovering mechanisms that regulate endogenous stem cell responses to selective cell death.

Hair cells

Hair cells are the primary sensory neurons of the auditory system and later line organ (in fish). In mammals, it has been assumed that lost hair cells are not replaced – thus, deafness due to hair cell loss is currently considered irreversible in humans. Intriguingly, a limited amount of regeneration has been seen in recent studies in mice [81,82]. In some nonmammalian systems, such as birds and fish, lost hair cells are readily replaced by surrounding cells called supporting cells [83–85]. Hair cells can be replaced by two distinct mechanisms: first, nonproliferative – direct transdifferentiation of support cells into hair cells [86] and/or, second, proliferative – mitotic expansion of support/progenitor cell pools and subsequent differentiation of progeny into hair cells [87,88]. Zebrafish larvae regenerate lateral line hair cells rapidly after damage, with almost all hair cells being replaced after 72 h [89]. Thus, zebrafish larvae are an excellent model system for applying chemical genetics to hair cell regeneration [90]. For instance, to explore cellular mechanisms involved in hair cell regeneration, Mackenzie and Raible inhibited cell division using a small-molecule inhibitor of microtubule assembly [83,91]. They demonstrated that blocking proliferation inhibited regeneration, in turn revealing that transdifferentiation could not compensate for disrupted support cell proliferation. Similarly, another group investigated if support cells underwent chromatin remodeling when transitioning from a quiescent to proliferative state [92]. Application of valproic acid and trichostatin A (TSA) showed that inhibition of histone deacetylase (HDAC) activity suppressed support cell proliferation, demonstrating the importance of HDAC in hair cell regeneration.

Small-molecule screens are particularly useful for exploring the role of developmental signaling pathways in regenerative processes. Several groups have utilized this approach to investigate the role of the Notch and Wnt pathways in hair cell regeneration [89,93]. For instance, following neomycin-induced hair cell ablation, pharmacological inhibition of Notch signaling (using the γ-secretase inhibitor DAPT) promoted SC proliferation and resulted in a concomitant increase in the number of regenerated hair cells [89]. The authors went on to show that DAPT acted specifically on a subpopulation of 'internal' support cells suggesting the existence of functionally distinct subtypes of support cells. In a similar study, Head *et al.* utilized a GSK3β inhibitor, 1-azakenpaullone (Az), to ask if Wnt activation could stimulate SC proliferation during hair cell regeneration. Following neomycin treatment, Az exposure led to elevated proliferation of support cells and an increase in the numbers of differentiating hair cells [93]. Collectively, these and related studies have demonstrated the power of chemical genetics for reveal critical insights into the regulation of regenerative process, such as hair cell regeneration.

Retinal cells

The retina, being an extension of the CNS, displays a woefully limited capacity for regeneration in mammals. Thus, the primary aim of cell-based retinal therapies is to provide the eye with functional replacements for cell types lost to disease or injury. This could be achieved either by transplantation of retinal neurons obtained by *in vitro* differentiation of stem cells, or by stimulating endogenous repair mechanisms. Although mammals do not have persistent retinal neurogenic sources, this capacity has been preserved in amphibians, chicks and fish. Four retinal stem cell niches have been described: first, the ciliary marginal zone (CMZ), a region at the circumferential perimeter of the retina that is responsible for annular growth but which normally does not contribute substantially to the regenerate; second, the retinal pigment epithelium (RPE), which in the birds and amphibians has been shown to undergo transdif-

ferentiation to give rise to new retinal cells; third, rod-committed progenitors, localized in the outer nuclear layer and believed to be committed to the rod photoreceptor lineage and fourth, Müller glia (MG), the primary glial cell type of the retina which responds to injury and currently represents a potentially conserved retinal stem cell across vertebrate organisms [94,95]. Another intriguing possibility for restoring vision, particularly relevant to chemical biology, involves a novel approach using light-activated photoswitch chemicals to convert retinal ganglion cells (RGCs) into transducers of light. This strategy has recently been applied to restore visual responses in blind rd1 mice lacking photoreceptors [96,97].

Intriguingly, although mammalian MG do not normally enter the cell cycle after retinal injury, in cell culture – or when treated with certain growth factors *in vivo* – (MG) appear to retain the potential for repair [98]. Primary human MG cells grown in defined culture conditions have been shown to differentiate into both photoreceptor cells and RGCs. Moreover, transplantation of rod photoreceptor precursors and RGC precursors derived from human MG cell cultures can successfully integrate into the host retina, restoring function in P23H rats exhibiting slow rod degeneration and in Lister hooded rats where RGCs were damaged by NMDA injection, respectively [99,100]. Immortalized MG cell lines derived from the adult human retina can also differentiate into retinal neurons [101]. On transplantation, these cells showed better migration in the neonatal retina of Lister hooded rats than into the dystrophic retina of the RCS rat indicating developmental cues may be critical for integration. These studies clearly indicate that human MG retains the capacity to replace lost retinal cells. Therefore, understanding how the regenerative potential of MG cells is regulated will be key to developing therapies seeking to stimulate endogenous repair mechanisms in the human eye.

Teleosts (ray-finned fishes) display a robust capacity to replace lost retinal neurons following a range of injury paradigms such as acute light lesion [102], surgical lesion [103] or cell-specific ablation [104–107]. Initially, progenitor cells in the outer nuclear layer were thought to be the only source of regenerating cells in teleosts. However, studies utilizing transgenic fish with GFP-labeled MG revealed that the primary injury-responsive retinal stem cell in the zebrafish was the MG [108–110]. Moreover, it was revealed that MG gives rise to outer nuclear layer progenitors, which are thought to be restricted to the rod cell lineage. Although MG are normally quiescent, responsible predominantly for maintaining general homeostasis, they can be induced upon injury to dedifferentiate to a stem-like state, reenter the cell cycle, and give rise to progenitor cells which differentiate to replace lost neurons. Unfortunately, in mammals, MG normally responds to injury by entering reactive gliosis [111]. Thus, retinal regeneration researchers are focused on delineating how dedifferentiated stem cell activation and reactive gliosis differ; to define mechanisms that stimulate beneficial versus deleterious MG responses to retinal injury. A series of excellent recent reviews have covered the current state of understanding of the MG stem cell niche [94,112,113]. Here, we will focus on how the use of chemical genetics has revealed important clues into how the regenerative potential of MG cells is controlled.

As Wnt signaling is central to numerous biological processes, Ramachandran *et al.*, investigated the role of β-catenin, the central signaling molecule in the Wnt signaling pathway, during retinal regeneration [114]. They observed that following a retinal stab wound, β-catenin, accumulated in the nucleus of MG and MG-derived progenitors. Using chemical genetics – pyrvinium (a casein kinase 1-α activator) or XAV939 (a tankyrase inhibitor) – to block β-catenin accumulation, resulted in a reduction in proliferation of MG-derived progenitors [115]. This suggested that β-catenin was required for the proliferation of retinal progenitors during regeneration. They further tested the role of Wnt/β-catenin by enhancing signaling using lithium chloride (LiCl), a GSK-3β inhibitor that prevents β-catenin degradation. In response to LiCl injection, the number of proliferating cells increased. Remarkably, LiCl injection stimulated proliferation in both the injured and uninjured retina, and progenitors in the uninjured retina gave rise to multiple retinal neuron subtypes.

Meyers *et al.*, further explored the role of Wnt/β-catenin in the CMZ during development and in MG cells during regeneration using an intense light lesion paradigm that limits cell loss to photoreceptors [116]. Using timed administration of a GSK-3β inhibitor (Az) during retinogenesis, they found that when Wnt activation was initiated at 36 h postfertilization (hpf), neuronal differentiation was blocked and progenitors were maintained in a proliferative state. Consistent with this, inhibition of Wnt (XAV939) at 3 days postfertilization (dpf) resulted in a loss of progenitor cell markers and a decrease in proliferation in the CMZ. However, exposure to Az at 6 dpf was not sufficient to induce proliferation in the central retina of larval fish, in contrast to injection of LiCl in adults. Finally, upon light lesion, treatment with Az from 1–5 or 3–5 days postlesion (dpl) led to a selective decrease in the number of proliferating MG cells (outer nuclear layer progenitor proliferation was unchanged). However, no effects were seen when treatments were limited to 0–3 dpl. This suggested that Wnt signaling is not required for MG activation but does

Key term

Sheddases: Enzymes that can cleave extracellular components of transmembrane proteins resulting in release of the ectodomain.

alter subsequent proliferation patterns. The authors went on to show that Wnt activation blocked asymmetric, self-renewing, divisions of MG following a light lesion, instead driving all daughter cells toward a progenitor fate and diminishing the number of MG cells. Together with Ramachandran *et al.*, the data suggest that Wnt/β-catenin plays a central role in controlling either dedifferentiation and/or proliferation of MG cells in zebrafish. In contrast, Zhu *et al.* recently showed that, in the chick, loss of nuclear β-catenin is correlated with proliferation of ciliary margin cells and RPE-derived progenitors following retinectomy [117]. Collectively, these studies highlight how differences between species, across injury paradigms, among alternative stem cell niches, and/or regarding the compounds used (with respect to chemical genetics), may have a profound impact on interpretations of the role specific signaling pathways play in regenerative processes.

To further characterize factors involved in retinal regeneration, Wan *et al.* hypothesized that MG secrete factors that stimulate their own dedifferentiation through activation of genes such as *ascl1a* [118]. To identify such factors, they screened for EGFR ligands that were upregulated following stab wounds. HB-EGF (heparin-binding epidermal-like growth factor) was the only ligand highly induced as early as 1 h postinjury. Knockdown of this gene led to a reduction in proliferating MG-derived progenitors while intravitreal injection of HB-EGF led to increased numbers of progenitors in the injured and uninjured retina, respectively. HB-EGF is released by ectodomain shedding, thus inhibition of sheddases by GM6001 (a pan metalloproteinase inhibitor) was used to further test the role of HB-EGF. GM6001 prevented proliferation of MG-derived progenitors following injury, suggesting that HB-EGF (or other factors activated by ectodomain shedding) was required for dedifferentiation. Since EGFR activation is associated with MAPK signaling [119], the authors investigated the role of EGFR signaling using pharmacological inhibitors of EGFR (PD153035), MAPK (ERK1 and 2 inhibitors, PD98059 and SL327). Their results demonstrated that inhibition of the EGFR signaling by any of these reagents reduced the number of proliferating progenitors by as much as 75%. This study highlights one of the potential drawbacks of chemical genetics; the lack of discrete downstream signaling molecules (e.g., the MAPK cascade and receptor tyrosine kinases) can cause ambiguity when using modulators of these factors. Nevertheless, applying multiple modulators, as exemplified by Wan *et al.*, can largely circumvent this issue.

As Notch signaling is an important regulator of retinoblasts during development, several groups have investigated whether Notch also impacts retinal regeneration. Following a stab wound injury, exposure to DAPT (an inhibitor of Notch/γ-secretase activity) led to an increase in proliferation at the injury site [118]. Similar observations were made using an improved γ-secretase inhibitor (RO4929097) following light lesioning of photoreceptors [120]. In addition, injection of RO4929097 to the uninjured eye was sufficient to stimulate MG proliferation (in contrast to DAPT) [118]. This is in keeping, however, with a study showing that sustained Notch activation is necessary to maintain glial fates in early postnatal MG cells in the developing mouse retina [121]. Thus, in fish, perhaps MG cells are predisposed to dedifferentiation with sustained Notch signaling being necessary to maintain their glial identity. In the stab wound paradigm, the effect of inhibiting Notch signaling could be negated by a loss in EGFR signaling, as MAPK or EGFR inhibition suppressed the effect of DAPT. In further studies, Ramachandran *et al.* demonstrated that *insm1a* was expressed in MG-derived progenitor cells and its suppression also resulted in an increase in the number of progenitors [122]. Therefore, the authors explored interactions between *insm1a* and Notch signaling. Using DAPT, they demonstrated that Notch was upstream of *insm1a*; exposure to DAPT abolished *insm1a* expression upregulation during regeneration. The authors argued that inhibition of HB-EGF expression by Notch-dependent *insm1a* upregulation may serve as a feedback mechanism to limit the zone of dedifferentiating MG cells. To test this, they inhibited EGFR signaling (PD158780) in an *imsm1a*-depleted retina, and showed that this prevented the expansion of MG-derived progenitor cells. These results suggest that interactions between HB-EGF, Notch and Insm1a define the zone of responsive MG cells at the site of stab wound retinal injuries.

The Jak/Stat (Janus kinase/Signal transducers and activators of transcription) signaling pathway is a transduction cascade for many growth factors and cytokines. Stat3 is expressed in MG cells following injury and, combined with Ascl1a immunolabeling, has been used to delineate three distinct populations of MG: Stat3-expressing Ascl1a-negative quiescent cells, Stat3-positive Ascli1a-positive proliferating cells and Stat3-negative Alsc1a-positive proliferating cells [123]. More recently, Zhao *et al.* utilized the stab wound model to explore roles for Jak/Stat signaling in MG activation during retinal regeneration. Chemical inhibitors of Jak/Stat signaling (JSI-124 and P6) sup-

pressed the generation of MG-derived progenitors in the injured retina by preventing induction of *ascl1a* expression. Timed exposures to inhibitors at 0–2 dpi and 2–4 dpi revealed that Jak/Stat signaling was not only critical for formation of progenitors but also for their expansion later on, demonstrated by a lack of BrdU-positive cells in injured fish retinas. These studies suggest that Jak/Stat signaling regulates MG activation and progenitor cell expansion during retinal regeneration.

Wan *et al.*, recently explored the role of insulin, IGF-1 and FGF signaling components in inducing MG cell dedifferentiation [103]. Insulin expression is increased in proliferating MG-derived progenitors following a stab wound injury, and knockdown caused a reduction in the number of proliferating MG-derived progenitors. Injection of insulin into the uninjured eye resulted in a dose-dependent increase in proliferation. Interestingly, synergistic effects were seen when ineffective concentrations of insulin and HB-EGF (or IGF-1 and FGF2) were injected as a pair. Based on the fact that knockdown of Igf signaling components (Igfra or Igfbp3) also resulted in a decrease progenitor cells, the authors explored the role of downstream signaling, PI3/Akt, using the pharmacological inhibitors LY294002 and PI-103. Both inhibitors reduced the number of proliferating progenitor cells postinjury, and when HB-EGF, insulin, or IGF-1/FGF-2 were injected into the uninjured eye – as did inhibition of MAPK (UO126) or β-catenin (pyrvinium). This last result is unexpected, as the molecules used to stimulate MG activation are not predicted to act through all four of the signaling cascades being pharmacologically inhibited. Moreover, it suggests that regeneration is dependent on coordinated interactions between all of the implicated pathways, thus rather than any one being sufficient for MG activation, crosstalk between them must be initiated when any singular path is exogenously stimulated. Nevertheless, this study exemplifies the power (and caveats) of applying chemical genetics to a complex multifactorial questions.

Besides responding to exogenous stimuli, MG also phagocytose dead cells. Bailey *et al.* explored the role of this particular function of MG in retinal regeneration using a light lesion model [124]. The authors used a chemical inhibitor of microglial phagocytosis called L-SOP and demonstrated that inhibition of phagocytosis reduced the number of proliferating MG cells and the number of regenerated cone photoreceptors. Since L-SOP is also an agonist of the metabotropic glutamate receptor (mGluR), they investigated if the effect on proliferation was mediated by mGluR using different agonists and antagonists. Their results indicated that the effect of L-SOP was not mediated by mGluR. L-SOP exposure did not affect Ascl1a or Stat3 expression in MG, suggesting that this chemical was acting either in parallel or downstream of Ascl1a activation. This work implicated a unique role for phagocytosis in regulating the response to retinal cell loss. One caveat of this study is whether phagocytic microglia also plays a role in modulating retinal regeneration, a possibility the authors felt was less likely based on normal distribution and morphology of microglia in L-SOP-treated retinas.

As described in the above examples, testing the role of suspected signaling pathways directly has been a powerful approach to increasing our understanding of regenerative biology. However, a major limitation is that it builds upon prior knowledge, thus biasing studies toward known targets and leaving less characterized pathways untested. A more general, nonbiased, approach can be achieved using forward chemical screens. This discovery-based strategy can provide valuable insights into any pharmacologically targeted factor impacting regenerative processes and thereby aid the development of therapeutic strategies as well.

Forward chemical genetics

Forward screening strategies provide an opportunity to discover novel effectors of biological processes of interest. Unlike reverse screens, no prior knowledge of underlying molecular mechanisms is necessary. Rather, such approaches are limited only by the availability of robust phenotypic assays amenable to miniaturization and/or compatible with large-scale screening strategies. The forward screening paradigm has therefore been essential for expanding our understanding of unknowns; critical for unveiling functional insight into factors and pathways that had either previously gone uncharacterized or unappreciated with respect to the biology of interest. Below we review some of the contributions that forward chemical screening has made to our understanding of regenerative biology.

Organismal regeneration

To our knowledge, although forward genetic screens have been applied to planarian regeneration, forward chemical screening has yet to be applied to organismal regeneration.

Appendage replacement: epimorphic regeneration

Taking advantage of the fact that the key players of regeneration are conserved between the adult and larval fin regeneration model, Mathew *et al.* performed a forward chemical genetics screen for modulators of caudal fin regeneration [125]. The ability to per-

form the assay in larval zebrafish enabled the use of microtiter culture (96-well plates); akin to common HTS assay formats. Accordingly, this was the first large-scale chemical screen involving a tissue regeneration paradigm. The compound library screened was comprised of 2000 bioactives and the US FDA-approved small molecules (MicroSource Discovery Systems). Libraries of existing drugs have several benefits: first, existing drugs are well characterized with regard to mechanisms of action (MoA), facilitating follow-up validations to identify target pathways; second, associated toxicities are known, focusing off-site analyses on organ systems likely to be impacted and; third, as a 'drug re-purposing' strategy it provides a potential fast track to clinical trials. After screening 32,000 amputated larval fish, 17 hits were implicated that inhibited fin regeneration. Interestingly, five of the hit compounds were glucocorticoids. This was the first evidence that activation of glucocorticoid signaling pathway can inhibit regeneration, demonstrating the potential of forward chemical genetics in tissue regeneration. Taking advantage of the ability to limit compound exposures to discrete time windows, Mathew *et al.* went on to demonstrate that a particularly potent glucocorticoid, beclomethasone dipropionate, was effective only when applied during the first 4 h after injury. This analysis revealed that glucocorticoid activation was targeting pathways critical for the formation of the wound epithelium and blastema – in other words, the initiation of a regenerative response – and not later stages involving stem/progenitor proliferation and differentiation.

In a related screen, Oppedal and Goldsmith screened for chemical inhibitors of caudal fin regeneration in adult zebrafish [126]. A total of 520 compounds were tested (a subset of the LOPAC 1280 library from Sigma), of which 13 were implicated in the primary screen and 2 were validated. Follow-up assays focused on an imidazoline receptor antagonist, AGN192403, as the implicated pathway was a potential novel modulator of regeneration. Tests with like-compounds confirmed the imidazoline receptor as the target and further analysis revealed that AGN192403 prevented blastema formation and proliferative outgrowth, but had no effect on initial stages of wound healing. Interestingly, the authors went on to show that prolonged exposure to AGN192403 (≥5 days postamputation) led to sustained disruptions in fin regeneration, even up to 2 weeks after washout. These studies reveal the potential of forward genetics to identify novel regulators of appendage regeneration, and reiterate the importance of chemical genetics for defining phase-specific roles for targeted factors/pathways in regenerative processes.

Tissue regeneration

Bone

To our knowledge, an *in vivo* model of bone regeneration compatible with large-scale chemical screening has not yet been described. However, small-molecules screens carried out in cultured cells with osteogenic potential have shown promising results. For example, Mundy *et al.* screened 30,000 compounds in a genetically modified immortalized murine osteoblast cell line in which BMP2 expression could be measured by luciferase assay [127]. They found statin, a drug known to lower cholesterol levels, could enhance osteoblast differentiation. This effect was further verified by increasing new bone formation in an *ex vivo* model, neonatal calvarial bone culture. In another example, Darcy *et al.* screened 5405 chemical compounds and found 45 that enhanced BMP2-induced osteoblast differentiation of myoblast cells [128]. Among them, two known anticancer immunosuppresants, rapamycin and FK-506, were further investigated. Both stimulated preosteoblast cells to differentiate into osteoblasts with or without BMP induction. Moreover, rapamycin countered the inhibitory effect of TGFβ1 on osteoblastogenesis. Both studies exemplify how existing drugs may have additional therapeutic benefits beyond original indications.

Naturally, discovering new drugs is also of great interest. In another cell study, Hojo *et al.* created a transgenic preosteoblast MC3T3E1 cell line expressing GFP under the regulation of a collagen type-1 promoter fragment; thus, GFP expression was correlated with osteogenesis. Using this tool, they screened 2500 natural and synthetic compounds, identifying an isoflavone derivative, glabrisoflavone, as an inducer of osteoblast differentiation independent of BMP, Runx2 and Wnt signaling. Glabrisoflavone is extracted from leaves of the licorice plant, *Glycyrrhiza glabra* [129]. Although extracts from *G. glabra* have been used in traditional medicines for many years, the specific biological function and molecular target(s) of glabrisoflavone remain unknown. Nevertheless, this study suggests a new application of this compound, and/or isoflavones in general, a finding clearly worthy of further investigation.

Monitoring stem cell commitment to the osteoblast lineage is another way to discover compounds contributing to osteogenesis. Alves *et al.* screened 1280 pharmacologically active compounds in mesenchymal stem cells and found five osteogenic factors targeting either Raf-MEK-ERK or the cAMP signaling pathways [130]. Another study similarly screened 1040 small molecules using an alkaline phosphatase assay to indicate osteogenesis. Thirty-six molecules were found to promote osteogenesis, while 20 compounds inhibit by increasing and decreasing the ALP activ-

ity [131]. Collectively, these screening studies highlight the advantages of applying forward chemical screening to the issue of bone repair, providing new applications for known drugs and discovering novel inroads into promoting osteogenesis.

Heart

Effective heart repair will require a deeper understanding of the regulation of myocardial differentiation, in particular with regard to the use of stem cell transplants. To discover new factors promoting myocardial fates, Sadek *et al.* screened 147,000 compounds using a pluripotent mouse stem cell line (P19CL6), leveraging the expression of the *Nkx2.5* gene as a marker of cardiovascular progenitor cells [132]. They identified a class of molecules, sulfonyl-hydrazone, competent for upregulating *nkx2.5* and other cardiac markers in a variety of stem cell types. Importantly, sulfonyl-hydrazone treatment of human mobilized peripheral blood mononuclear cells (M-PBMCs) induced increased expression of cardiac marker genes. Human M-PBMCs represent perhaps the most promising stem cell source for cardiac repair, thus showing pharmacological conservation of sulfonyl-hydrazone effects in this cell line enhances the potential for therapeutic benefit. Together, the data support sulfonyl-hydrazone small molecules as promising drugs for promoting cardiac repair.

Using an *in vitro* system, Uosaki *et al.* screened 280 kinase inhibitors on mouse embryonic stem cell-derived cardiomyocytes [133]. The initial screening identified nine chemicals that impacted cardiomyocyte proliferation, modulating four corresponding kinase signaling pathways: inhibitors of GSK-3, MAPK or CaMKII and activators of ERK. In another *in vitro* screen, Wnt was identified as a key regulator of heart regeneration when fluorescently labeled human embryonic stems cells were treated with 550 modulators of known pathways and analyzed using high-throughput imaging [134]. It should be noted that although these studies only examined small molecules targeting known pathways, these tools have potential to be expanded to the large-scale screening to search for wide range of chemicals.

As discussed above, degenerative and autoimmune disorders are often linked to the promise of stem cells. A better understanding of how the regenerative potential of endogenous stem cell niches is regulated at the molecular level will aid efforts to develop reparative therapies for these conditions. Unfortunately, most cellular regeneration paradigms represent the proverbial 'black box' in this respect. Forward screens therefore provide perhaps the best option for bringing cellular regeneration – the replacement of specific disease-relevant cell types – to light.

Cellular regeneration

Hair cells

Recent studies have revealed supporting cells as the source of regenerating hair cells and reverse chemical genetics has been useful for delineating roles of canonical development signaling pathways, such as Wnt and Notch (see above). Namdaran *et al.* adopted an unbiased forward screening strategy to discover novel small-molecule modulators of support cell proliferation following neomycin-induced hair cell loss [135]. Screening 1680 FDA-approved drugs resulted in the identification of both inhibitors and enhancers of hair cell regeneration. This study implicated glucocorticoids, such as dexamethasone and prednisolone, as enhancers of hair cell regeneration; this is in contrast to previous fin regeneration studies in which glucocorticoid agonists had an inhibitory effect [125]. In addition, glucocorticoid treatment, in the absence of hair cell loss, also stimulated an increase in hair cell numbers. Furthermore, as other anti-inflammatory compound classes failed to have an effect on hair cell regeneration, their data suggested glucocorticoids may be acting via mechanisms other than immunosuppression – perhaps as direct neuroprotectants. Lastly, this study also discovered novel inhibitors of hair cell regeneration, such as topotecan (an inhibitor of topoisomerase activity), which acted by preventing support cell proliferation, and estrogen receptor antagonists which delayed hair cell regeneration by reducing support cell proliferation.

In a similar study, Moon *et al.* screened 470 compounds for modulation of hair cell regeneration in zebrafish larvae [136]. Using several transgenic lines to facilitate screening via confocal microscopy, they identified 20 compounds that enhanced regeneration by more than 25%. Their screen identified LMWF (low-molecular-weight fucoidan), a natural product present in brown seaweed as an enhancer of regeneration. Based on the fact that Notch and FGF signaling also impact hair cell development and regeneration, Moon *et al.* utilized pharmacological inhibitors of Notch and FGF to test the role of these signaling pathways and to determine if coexposure with LMWF could alter their capacity to influence regeneration [89,136,137]. Their studies showed that LMWF did not improve the capacity of DAPT to enhance regeneration, indicating that the effect of LMWF is not synergistic with Notch signaling. However, LMWF could rescue the delayed regeneration phenotype induced by FGF inhibition, suggesting that LMWF targets are downstream or dominant over FGF. Further studies are required to characterize the molecular mechanism of action of the LMWF.

Skeletal muscle

Although skeletal muscle has an inherent ability to regenerate, regenerative capacity decreases with age [138] and with muscular dystrophy. Muscular dystrophy is a genetic disease caused by mutations in muscle proteins resulting in muscle degeneration [139]. The most common form of this disease is Duchenne muscular dystrophy (DMD), caused by mutations in the gene dystrophin [140]. In DMD patients, muscle stem cells known as satellite cells are depleted with age, possibly due to sustained activation and impaired renewal, resulting in increased muscle degeneration [141]. In zebrafish, disrupted skeletal muscle structure can be easily examined using a birefringence assay, measuring the degree to which light is skewed when going through the normally arrayed structure of the sarcomeres. To identify potential therapeutics for DMD, Waugh *et al.* performed a forward screen for chemicals that can restore normal birefringence in a mutant zebrafish model of DMD [142]. Screening 640 largely FDA-approved compounds resulted in the identification of six hits, three of which were classified as monoamine agonists. A secondary screen of additional monoamine agonists (and serotonin) determined that fluoxetine, a selective serotonin reuptake inhibitor, provided the strongest rescue phenotype (other than serotonin itself). Microarray expression studies suggested that fluoxetine may act by sustaining calcium homeostasis, and disruptions in this pathway have been previously implicated in DMD.

A similar study, by Kawahara and Kunkel was conducted in *sapje* and *sapje-like* mutants [143]. They screened a library of 1120 compounds, the majority being FDA-approved. Using the same birefringence assay, they identified seven hits that restored a normal muscle phenotype. Out of the seven identified hits, aminophylline, a nonselective phosphodiesterase inhibitor, had the strongest effect. Aminophylline was known to increase intracellular cAMP levels leading to activation of cAMP-dependent protein kinase (PKA), and increased activation of PKA was detected in treated fish. These studies exemplify how combining genetic mutants with forward chemical screens can be used to derive novel insights into stem cell biology and to further the development of reparative therapeutics for patients.

Pancreatic β-cells

The target of β-cell regeneration studies is to increase β-cell mass as a potential therapy to treat diabetes. Molecular regulators of β-cell proliferation and differentiation have not been fully characterized, although human β-cell replication has been clearly observed in response to metabolic demand, such as in obesity or during pregnancy [144]. Therefore, identification of chemicals that increase β cell production will enhance our understanding of molecular mechanisms that represent potential therapeutic solutions for diabetes.

In order to identify modulators of β-cell regeneration, Andersson *et al.* screened 7186 compounds (including FDA-approved drugs, natural products and uncharacterized entities) in a transgenic zebrafish model enabling selective ablation of β cells [145–147]. After screening ~100,000 larvae, five compounds were identified that doubled the number of regenerating β cells. Remarkably, four of the five hit compounds were predicted to act by enhancing the adenosine signaling. Critically, adenosine signaling was validated in a mouse model of diabetes, promoting β-cell proliferation. Equally important, the activity of the most effective adenosine agonist, NECA, faded when normal glucose levels were achieved. Hence, large-scale *in vivo* chemical screening identified adenosine signaling as a novel pathway for promoting increased β-cell mass. Annes *et al.* also identified adenosine kinase inhibitors (ADK-Is) as promoters of β-cell replication in mammalian tissue culture [148]. They screened ~850 compounds for increased numbers of PDX1/Ki67-positive β cells in primary rat islet cultures. They identified two hits and determined that effect of one, ADK-Is, was mediated by the mTOR pathway and, moreover, was specific to β cells. The adenosine pathway is known to provide cytoprotective effects as an anti-inflammatory agent and can promote repair in a variety of tissues [149]. Taken together, these data suggest that inflammation may function to inhibit β-cell proliferation, and provides a promising new therapeutic target for diabetes. In summary, whole-organism and *in vitro* screens converged on the adenosine signaling pathway as a promising therapeutic target for restoring β-cell mass in diabetic patients.

The screen performed by Andersson *et al.* was not biased toward any specific cellular mechanism for increasing β-cell numbers and hence allowed discovery of a broad range of hit compounds [145]. A more targeted screen for modulators of β-cell differentiation was performed by Rovira *et al.*, using transgenic lines to identify small molecules that induced premature secondary islet formation [150]. They screened a library of 3131 compounds consisting mainly clinically approved drugs (the Johns Hopkins Drug Library, JHDL) and identified six compounds promoting endocrine cell differentiation. Follow-up MoA studies on the compounds, mycophenolic acid and tetraethylthiuram disulfide, revealed two novel targets involved in β-cell differentiation, GTP and RA, respectively. Using a series of alternative modulators, they showed that inhibition of GTP or RA synthesis was sufficient to induce secondary islet formation. Investigating another

means of increasing β-cell mass, Tsuji *et al.* performed a high content screen in transgenic larval zebrafish to identify compounds stimulating β-cell replication [151]. Screening a library of 833 compounds resulted in the identification of 20 hits. Interestingly, all hits fell into one of three categories: stimulators of retinoid acid signaling, enhancers of serotonergic signaling or glucocorticoid receptor ligands. The effect of glucocorticoids were found to be indirect (i.e., increased glucose levels), while serotonin and RA signaling appeared to have direct effects (i.e., no effect on glucose levels) – albeit these results were based on a single representative compound from each category. Representative hits were further tested for effects on β-cell regeneration using a Type I diabetic model involving selective β-cell ablation [146,147]. Both RA and prednisolone enhanced β-cell regeneration, while the serotonin reuptake inhibitor, trazodone, had no effect. Since all three serotonergic compounds implicated in the original assay induced only a relatively mild amount of β-cell proliferation, the authors concluded that compounds promoting robust proliferation can also lead to enhanced regeneration.

In an effort to more comprehensively screen for chemicals that increase β-cell mass, we recently completed the first quantitative high-throughput screen (qHTS) in a vertebrate model organism [152]. We adopted existing HTS instrumentation (a microtiter plate reader) to reporter-based assays in larval zebrafish; a methodology termed ARQ*iv* (Automated Reporter Quantification *in vivo*) which we developed to harness the full potential of zebrafish for whole-organism drug discovery [99]. ARQ*iv* can screen fish at true high-throughput rates, in turn, allowing us to apply HTS best practices, such as qHTS; in other words, titrating all compounds across a six- to eightfold dilution series in the primary screen to reduce false hit-call rates [153]. Using a robotized iteration of the ARQ*iv* platform and the JHDL for qHTS, we screened over 500,000 larval zebrafish, implicating a total of 177 drugs as stimulators of β-cell differentiation and/or proliferation. To date, 24 of 39 drugs rescreened have been validated, and MoA follow-ups have revealed another novel regulator of β-cell differentiation, the NF-κB signaling pathway. The data further suggest that serotonergic signaling stimulates β-cell proliferation selectively, without altering the proliferation of other endocrine cell subtypes, and also stimulates β-cell replication in mice. These findings have important therapeutic implications as increasing β-cell mass in a cell-type specific manner could have significant benefits for diabetic patients (e.g., reduced side effects).

In summary, forward chemical screens have identified several novel pathways for promoting β-cell differentiation or to stimulate β-cell proliferation. Both end points are useful, for instance, compounds that induce endocrine cell fate could be used to guide cell fate in stem cell cultures. More intriguingly, recent findings suggest that existing β cells proliferate to maintain homeostasis and during regeneration in mammalian models, thus compounds that stimulate β-cell proliferation represent a promising new therapeutic strategy for diabetic patients. The screens discussed above, and many others which we were unable to summarize in detail (see Table 1), provide examples of the power of forward genetics for revealing new insights into regenerative biology and for aiding the development of regenerative therapies.

Conclusion & future perspective

In this review, we have discussed how chemical genetics can reveal key molecular components and/or pathways that regulate regeneration. Reverse genetic strategies can resolve the role of known molecular pathways, while forward screens represent a more exploratory approach for revealing novel targets. Within the context of chemical genetics, both methods facilitate the understanding of regenerative mechanism, thus assist the design of new therapeutics. Supported by recent advances in computer-assisted automation and robotics, as well as robust quantitative assays, whole-organism high-throughput drug screening is well-positioned to drive the discovery of novel insight into regenerative processes. Several rapidly developing technologies have the potential to enhance the power of whole-organism chemical screening for regenerative biology. First, CRISPR-based gene editing enables the development-sophisticated disease models in regenerative species useful for both reverse and forward chemical genetics. Second, microfluidics and femtosecond laser surgery [154] will provide a powerful platform for adapting more regenerative animal models to HTS. Third, automated microscopy facilitates image-based 'high content' screening of animal models in multiwell and/or microfluidic formats. Finally, recent advances in the synthesis of tagged libraries facilitate compound pooling and/or targeting of more than one pathway [155]. In summary, chemical genetics has made significant contribution in moving the field of regeneration biology forward. We expect that the combination of recent technological breakthroughs with small-model species enabling *in vivo* HTS will accelerate progress in regenerative biology research.

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Executive summary

- Chemicals targeting biochemical and biophysical signaling can provide valuable insights into regeneration at organismal, tissue and cellular levels.
- Chemical genetics has the power to dissect out the stage-specific roles for molecular pathways during regenerative processes.
- Several strengths of chemical genetics: temporal control, graded responses and the ability to circumvent genetic redundancy and/or compensation, are extremely useful for studying highly dynamic processes such as regeneration.
- While reverse genetics helps to define/validate roles of known molecules, forward genetics can implicate the unknown and therefore has a greater potential to provide insight into novel regulators of regenerative biology.
- Whole-organism quantitative high-throughput screening (qHTS) for enhancers and repressors of regeneration can speed the pace of discovery in the field of regeneration.

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