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TOPICAL REVIEW

Pharmacological Inhibition of Protein Lipidation

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Abstract Lipid modifications of mammalian proteins are widespread, modifying thousands of targets involved in all aspects of cellular physiology cellular physiology. Broadly, lipidations serve to increase protein hydrophobicity and association with cellular membranes. Often, these modifications are absolutely essential for protein stability and localization, and serve critical roles in dynamic regulation of protein function. A number of lipidated proteins are associated with diseases, including parasite infections, neurological diseases, diabetes, and cancer, suggesting that lipid modifications represent potentially attractive targets for pharmacological intervention. This review briefly describes the various types of posttranslational protein lipid modifications, proteins modified by them, and the enzymatic machinery associated with these. We then discuss several case studies demonstrating successful development of lipidation inhibitors of potential (and more rarely, realized) clinical value. Although this field remains in its infancy, we believe these examples demonstrate the potential utility of targeting protein lipidation as a viable strategy for inhibiting the function of pathogenic proteins.

Keywords Protein lipidation · Post-translational modification · Subcellular localization · Membrane binding · Lipid raft · Microdomain

Overview of Protein Lipidation

Protein modifications are ubiquitous mechanisms for regulation of protein structure, activity, and localization. Many of these modifications (e.g., disulfide oxidation) are co-translational, serving a necessary role for folding the protein into its native conformation, and remain with the protein for its functional lifetime. A smaller, but still significant, fraction are reversible modifications that are added and removed many times before a protein is degraded, and therefore play a key role in rapid, reversible regulation of protein function. Among dozens of different chemical moieties used for protein modification, a small subset serves to increase protein hydrophobicity by attaching lipid anchors: fatty acids, isoprenoids, sterols, and/or phospholipids. The common outcome of such lipid modifications is increased affinity for cellular membranes; however, they can also facilitate intra and intermolecular protein–protein interactions and confer stability to the modified protein (Resh 2013). The various lipidations are carried out by distinct enzymes at distinct cellular locations, ultimately conferring distinct properties to the modified proteins. The modifying enzymes can be cytoplasmic (S-prenylation and N-myristoylation) or membrane-bound (S-palmitoylation) (Hang and Linder 2011), and modify proteins on the cytoplasmic face of the membranes (myristoylation, S-palmitoylation, and prenylation) or in the lumen of the secretory pathway (GPI anchor). Moreover, lipids modifications can serve as guides for targeting of proteins to specific membrane subdomains, with saturated fatty acids (incl. GPI-anchors and palmitate) and sterols potentially targeting the tightly packed 'lipid raft' environment, while unsaturated and branched hydrocarbon anchors (e.g., in prenylated proteins such as G-protein βγ, K-ras 4B, Rab family proteins) favor the less ordered non-raft membrane regions (Levental et al. 2010).

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A large number and broad variety of disease-relevant proteins are lipid modified, with these modifications often critical for protein function, suggesting that pharmacological intervention of protein lipidation may be an efficient—although currently underexplored—approach to drug discovery. In this review, we will briefly introduce the various post-translational protein lipidations with a focus on disease-associated proteins, and discuss several case studies of drugs designed to interfere with these modifications.

Cholesterylation

Cholesterol is an abundant plasma membrane lipid in eukaryotes and is therefore vital for membrane physiology. In addition, it serves as a precursor for a variety of steroid-based endocrine signaling molecules (e.g., glucocorticoids, bile acids, and estrogens) and is also used, albeit infrequently, for protein modification. Remarkably, to our knowledge, there remains only a single well-characterized example of a protein being modified by cholesterol: the patterning morphogens of the Sonic Hedgehog (Shh) family (Lewis et al. 2001). During biosynthesis, these proteins undergo autocatalytic cleavage wherein a catalytic thiol attacks the carbonyl of the preceding amino acid, resulting in the formation of a thioester, which then reacts with cholesterol as a nucleophile to produce a cholesterol-modified N-terminal fragment. This lipidated fragment is the signaling-active, secreted form of the protein. Such cholesterol modification is a critical regulator of the spatial restriction/localized signaling effects of these proteins (Porter et al. 1996). Although the Hedgehog family is the only one that has been definitively identified to be modified in this way, chemical tagging approaches suggest many others remain to be detailed (Heal et al. 2011; Hulce et al. 2013).

Glycophosphatidylinositol Anchors

Glycophosphatidylinositol-anchored proteins (GPI-AP) constitute ~0.5 % of all proteins in eukaryotes (Eisenhaber et al. 2001). The GPI anchor is comprised of a diacyl phosphatidylinositol lipid covalently coupled to a trimannosyl–glucosaminyl glycan via a phosphodiester, then to the protein via a C-terminal ethanolamine. Assembly of the anchor and transfer to the protein occur in the endoplasmic reticulum (ER), mediated by nearly two-dozen different enzymes. GPI-modified proteins are transferred from the ER to the plasma membrane via the Golgi, where they are exclusively localized in the extracellular leaflet (Magee 1990). In this membrane, the GPI anchor is believed to confer specific association with specialized membrane microdomains known as lipid rafts, because of the preferential interactions between its saturated acyl chains and the ordered environment of the membrane raft (Mayor and Riezman 2004). Finally, a functionality unique to GPI-anchored proteins in lower and higher eukaryotes is that they can be released from the cell surface by cleavage of the lipid anchor by phosphatidylinositol-specific endogenous hydrolases (Chatterjee and Mayor 2001). Such phospholipases show specificity for certain GPI-APs (Muller et al. 2012), for example, phospholipase C acts on acetylcholinesterase (Low and Finean 1977) and phospholipase D on alkaline phosphatase (Low and Prasad 1988). By controlling the release of GPI-APs from the membrane, these enzymes play a critical regulatory role in their expression and function (Sharom and Lehto 2002).

Myristoylation

Approximately 0.5–0.8 % of all eukaryotic proteins are covalently modified with myristate, a 14-carbon saturated fatty acid. For this modification, an amino peptidase cleaves the initiator methionine to expose an N-terminal glycine (part of a (GXXXS/T motif) that gets myristoylated through a stable amide linkage by one of two (in humans) cytosolic *N*-myristoyl transferases (NMT1 and 2). Myristoylation is usually co-translational, but can also occur post-translationally, when caspase cleavage activated by apoptosis exposes a cryptic myristoylation site in previously unmodified proteins (Chen and Manning 2001).

The main function of myristoylation appears to be membrane targeting; however, myristate is believed to be generally insufficient to stably anchor protein to membranes. Rather, this modification is hypothesized to be one part of a two-signal mechanism, wherein myristate confers weak membrane binding, which is then stabilized by a second signal, such as:

1. A polybasic stretch of amino acids that interacts electrostatically with the negative charged phospholipids (phosphatidyl serine or phosphatidyl inositol) prevalent on the cytosolic face of many cellular membranes (e.g., c-Src, HIV-gag).
2. Hydrophobic residues proximal to the myristoylation site (small G-protein Arf1).
3. An additional lipid modification, such palmitoylation (Src-family kinases and Gα-subunits).

Myristoylation offers an interesting example of regulation of membrane binding without post-translational control of protein modification, via the “myristoyl switch”. Myristate is buried in a hydrophobic cleft in certain proteins and its exposure is regulated by ligand binding or protein multimerization. An example is the HIV matrix protein myr-MA, which guides the intracellular localization of HIV-Gag proteins to membrane rafts during viral assembly, and its dissociation during infectivity using the “myristoyl switch" mechanism. The equilibrium between the 'myr-exposed' and 'myr-sequestered' states is entropically driven: self-associating Gag subdomains promote the exposed state, while the cleavage of Gag by HIV-1 protease triggers the sequestered state, causing the release of the protein from the membrane (Tang et al. 2004).

Prenylation

The most abundant and evolutionarily conserved proteins modified by prenylation are small GTPases, including those of the Ras and Rab families. This modification involves the posttranslational addition of a C_{15} (farnesylation) or C_{20} (geranyl-geranylation) isoprenoid via a thioether to the C-terminal cysteine of a cytosolic protein [detailed review in Zhang and Casey (1996)]. The recognized signal for protein prenylation is known as the 'CAAX box', a C-terminal motif featuring the prenylated cysteine, two aliphatic amino acids, and a variable amino acid (usually serine, methionine, cysteine, alanine, glutamine or leucine). This variable terminal residue of the CAAX box is a determinant of the choice between the two types of prenylation, e.g., if X is leucine and if the CAAX box has a proximal polybasic domain enriched in lysine and arginine, the protein tends to be geranyl-geranylated (e.g.,, Rap, Rho, Rac) (Zhang and Casey 1996). Prenylation is carried out by heterodimeric enzymes, either Farnesyl Transferase (FTase) or one of two geranyl–geranyl transferases (GGTase-I and -II), and used for recruitment of otherwise soluble proteins to the cytoplasmic leaflet of the ER. Here, the Ras-converting enzyme (Rce1) proteolytically removes the AAX terminal motif, followed by carboxymethylation of the exposed C-terminal cysteine by isoprenyl cysteine carboxy methyl transferase (ICMT) (Zhang and Casey 1996). The complete processing pathway is required for the production of stable prenylated proteins.

It is believed that a C_{20} geranyl–geranyl group is sufficiently hydrophobic to autonomously anchor a protein to membranes, whereas farnesyl is not (Bhatnagar and Gordon 1997). Some geranyl–geranylated proteins—e.g., the small GTPases of the Rho family—are regulated by a similar mechanism to the "myristoyl switch", except that the hydrophobic pocket that sequesters the lipid anchor exists in a separate protein, a Rho GDP-dissociation inhibitor (Rho-GDI) (Nomanbhoy et al. 1999). For Rho, burial of its lipid anchor in a hydrophobic pocket of Rho-GDI is sufficient to prevent its association with membranes, thereby preventing interaction with membrane-resident GTP-exchange factors (GEFs) and subsequent activation. Farnesylated proteins require a second signal for stable membrane interaction, as is the case for the proto-oncogenes of the Ras family (Hancock et al. 1990). This two-signal paradigm allows reversible tuning of membrane association, which can then be used for regulation of subcellular localization (Rocks et al. 2005).

Palmitoylation

While the lipid modifications discussed thus far have well-defined sequence specificities that mark their target proteins and relatively narrow substrate groups, palmitoylation is both less specific and broader. Acyl biotin exchange and proteomic analyses have annotated hundreds, if not thousands, of different palmitoylated proteins (Martin et al. 2012; Ivaldi et al. 2012; Martin and Cravatt 2009; Merrick et al. 2011; Ren et al. 2013; Yount et al. 2010), and despite this wealth of data, no obvious sequence motifs specifying palmitoylation have emerged. There are three variations of protein palmitoylation, S-, N-and O-palmitoylation, with S-palmitoylation being the most widely explored (Tables 1, 2).

S-palmitoylation

S-acylation involves the addition of a fatty acid onto proteins via cysteine thioesterification. Although a number of fatty acids [including stearate, oleate, and even polyunsaturated fatty acids (Webb et al. 2000)] can be incorporated by this mechanism, the most common is 16-carbon, saturated palmitic acid, thus S-acylation is often referred to as 'palmitoylation'. Unlike other lipidations that exclusively modify otherwise soluble proteins, palmitoylation modifies both peripheral membrane proteins and transmembrane ones. Another distinguishing feature of palmitoylation is that it is reversible, thus serving as a mechanism to shuttle substrate proteins between the cytosol and the membranes (Rocks et al. 2010), or between coexisting membrane domains (Levental et al. 2010). Palmitoylation is often observed as a second lipid modification, imparting stable membrane attachment to proteins transiently interacting with membranes through myristoyl or prenyl groups; however, single palmitoylation and multiple palmitoylation of a single protein are also quite common.

S-palmitoylation is mediated by a family of cysteine-rich domain (CRD)-containing palmitoyl acetyl transferases (PAT) termed DHHC for a consensus Asp-His-His-Cys sequence in their active site. This DHHC PAT motif is conserved from yeast to primates, the latter possessing 23 such enzymes with seemingly overlapping specifies (Ohno et al. 2012; Greaves and Chamberlain 2011). DHHCs are multi-pass membrane proteins having 4–6 transmembrane domains (TMD) and are broadly distributed in cellular membranes, including the ER, Golgi and the plasma membrane (PM). Transfer of the palmitate from the donor

Table 1 Summary of lipid modifications of proteins

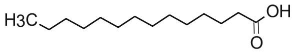
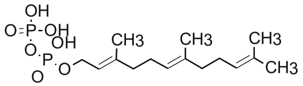
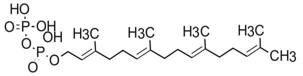
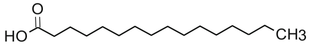
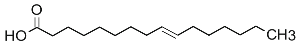
Lipid modification	Modifying group	Attachment/consensus motif	Linkage	Modification type	Modifying enzyme(s)	Stability	Examples
N-myristoylation	C14 Myristate 	N-terminal Glycine MGXXXS/T	Amide	Co-translational	*N*-myristoyl transferase (NMT1 and 2)	Stable	c-Src, HIV-gag
Prenylation	Isoprenoid C15 (farnesyl) pyrophosphate 	C-terminal Cysteine CAAX box	Thioether	Co-translational	Farnesyl transferase (FTase)	Stable	HRas, NRas, K-Ras, Cdc42, CENP-E, CENP-F, RhoB, Rheb
	C20—geranyl–geranyl pyrophosphate 	CAAL CC/CXC	Thioether	Co-translational	Geranyl–geranyl transferase (GGTase I and II)	Stable	Rab, Rho GTPases
S-acylation	Palmitate (C16) 	Cysteine	Thioester	Posttranslational	Palmitoyl transferases (PATs) Acyl protein thioesterases (APTs)	Labile	H-Ras, N-Ras, LAT, Fyn, Lck
N-palmitoylation	Palmitate (C16) Palmitoleate 	Cysteine Serine	Amide oxyester	Posttranslational	MBOATs HHat Porcin		Hedgehog, Wnt3a
Glycophosphatidyl inositol (GPI)	Phosphatidyl inositol		Amide	Co-translational	Many enzymes Phospholipase C	Labile	Acetylcholineesterase Placental alkaline phosphatase

Table 2 Summary of small molecule modulators of protein lipidation

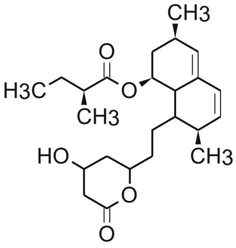
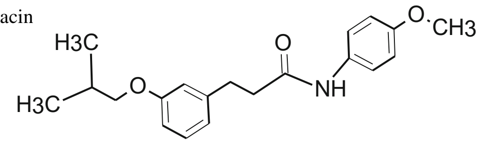
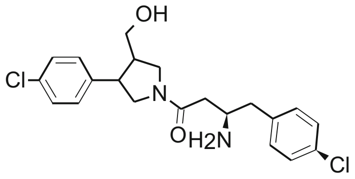
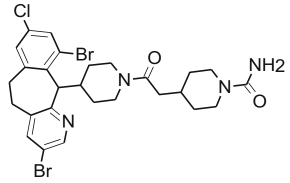
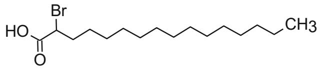
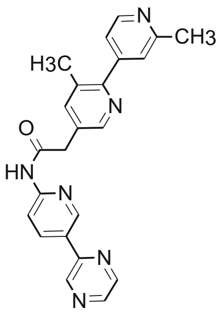
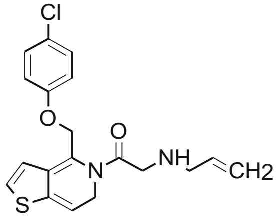
Structure		Primary target	IC_{50} (μM)	Mechanism
Lovastatin		HMG-CoA reductase	0.003–0.011	Interferes with cholesterol and isoprenoid biosynthesis by inhibiting the rate-limiting enzyme in the mevalonate pathway (Morck et al. 2009)
Gepinacin		Gwt1 (fungal acytransferase)	5–10	Inhibits a critical acyltransferase in the biosynthetic pathway of fungal GPI anchors (McLellan et al. 2012)
PF-03402623		*N*-Myristoyl transferase (*Leishmania donovani*)	0.093	Species-selective inhibition of *Leshmania donovani* NMT with demonstrated selectivity over human and plasmodium species (Hutton et al. 2014; Bell et al. 2012)
Lonafarnib		Farnesyl transferase	0.002	Blocks the posttranslational attachment of the prenyl moiety to C-terminal cysteine residue of prenylated proteins by inhibiting the enzyme farnesyl transferase (End et al. 2001)
2-Bromopalmitate		Over 450 targets that include both palmitoylated and non-palmitoylated proteins	10–15	A "suicide inhibitor" with promiscuous effects on over 450 targets that include both palmitoylated and non-palmitoylated proteins (Davda et al. 2013)
LGK947		PORCN	0.0004	Dysregulates Wnt signaling, by interfering with PORCN, an MBOAT family *O*-acetyl transferase, responsible for its palmitoleation (Liu et al. 2013)

Table 2 continued

Structure		Primary target	IC_{50} (μM)	Mechanism
RU-SKI 41		Hhat	0.18	Inhibits the palmitoylation of Shh by targeting its palmitoylating enzyme Hhat, an MBOAT family *O*-acetyl transferase (Petrova et al. 2013)
GO-CoA-Tat		GOAT	5	Bi-substrate (octanoyl CoA and ghrelin) analog peptide that selectively inhibits the octanoylation of ghrelin by GOAT (Barnett et al. 2010)

(palmitoyl-CoA) to the protein proceeds via an acyl enzyme intermediate (Dietrich and Ungermann 2004). Palmitoylation is also regulated by the oligomerization status of the DHHC enzymes. Specifically, DHHC2 and DHHC3 were found to exist in equilibrium between monomer and homo/heterodimeric states, with the monomers as the more active form (Lai and Linder 2013). Perhaps because of the multiplicity of enzymes mediating palmitoylation, there is no single consensus motif to indicate a palmitoylation site, although such sites can be predicted using algorithms based on the context in which the modified cysteine residue is present (Ren et al. 2008; Kumari et al. 2014), e.g., a cysteine in a transmembrane domain at the interface between the cytoplasm and the membrane. Depalmitoylation is even less understood, with three possible palmitoyl protein thioesterases (PPTs) described: the acyl protein thioesterases (APT1 and 2) and palmitoyl protein thioesterase (PPT1) (Das et al. 1997; Soyombo and Hofmann 1997; Camp et al. 1994). The latter is a lysosomal lumen hydrolase that nonspecifically deacylates C14-18 acyl side-chains (myristate, palmitate, stearate, and oleate) in vitro. Thus, there are only two known cytoplasmic thioesterases compared to 23 palmitoyl transferases, suggesting that the former have broader specificity, while the latter regulate the dynamic palmitoylation of specific substrates (Chavda et al. 2014).

The increased membrane affinity of lipidated proteins affects their localization and function. For example, dynamic cycles of palmitoylation via PATs and de-palmitoylation via thioesterases enable temporal cycles of membrane association and dissociation within the cell, which act as an organizing system for subcellular localization of peripheral proteins (Rocks et al. 2010). A prominent example is the continuous acylation and deacylation cycles of H-Ras and N-Ras that are required for the dynamic shuttling of these important oncogenes between cellular locations. According to a kinetic trapping model, prenylation of these proteins promotes transient interactions with membranes, allowing them to sample all cellular membranes. Palmitoylation then traps the protein in the membrane, thus fixing the location of the lipidated protein at the subcellular localization of its specific PAT, which in the case of Ras is the Golgi Apparatus, from which Ras is delivered to the PM by vesicular traffic (Rocks et al. 2005; Goodwin et al. 2005). In concordance with this model, interfering with either palmitoylation or depalmitoylation causes Ras mislocalization (Dekker et al. 2010) and perturbation of mitogenic activity (Cuiffo and Ren 2010).

N- and O-palmitoylation

In addition to the cholesterol-mediated proteolytic processing described above, the secreted morphogens of the Hedgehog (Hh) family are modified with palmitate. However, distinct from S-acylation, the palmitic acid is attached to the N-terminus rather than a cysteine thiol group. This reaction is mediated by an enzyme called Hedgehog acetyl transferase (Hhat), a multipass membrane protein of the MBOAT family (membrane-bound *O*-acyl transferase), and is required for the formation of large soluble Hh oligomers and the resulting functionality of the Hedgehog proteins (Chen et al. 2004). Another MBOAT family member, Porcupine, is responsible for palmitoylation of several of the ubiquitous secreted morphogens of the Wnt family, although here the palmitoylated residue is a serine, and thus, the modification is an O-acylation (Willert et al. 2003). As for Hh, lipidation is necessary for protein function, as interference with palmitoylation also blocks Wnt secretion (Gao et al. 2011).

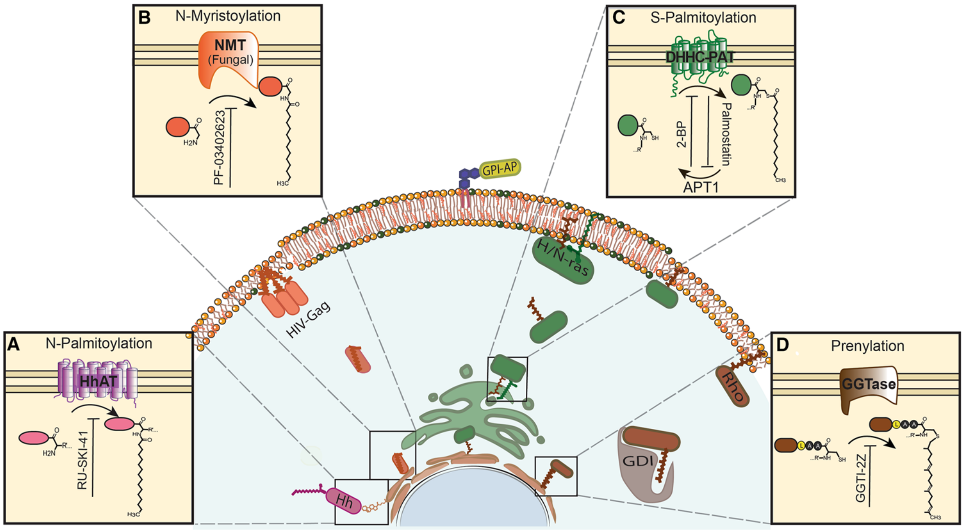


Fig. 1 Protein lipidation and its pharmacological inhibition. **a** N-palmitoylation of Hedgehog (Hh), which is also cholesterylated, can be inhibited by targeting the enzyme Hedgehog acetyl transferase (HhAT) with RUSKI-41. **b** Myristate can reversibly target proteins to membranes via a "myrisotyl switch". This process can be targeted in pathogenic parasites by inhibiting their *N*-myristoyl transferase (NMT), e.g., by PF-03402623. **c** S-palmitoylation is a widespread, reversible lipidation that recruits proteins (e.g., the oncogenes N-/H-ras) to membranes and ordered membrane subdomains known as rafts. Palmitoylation is the only reversible lipid modification: the forward reaction is catalyzed by a family of DHHC palmitoyl transferases (PATs) while depalmitoylation is mediated by one of three known protein thioesterases (APT1/2 and PPT1). PATs are inhibited nonspecifically by 2-bromopalmitate (2-BP) while depalmitoylation can be affected by palmostatins. **d** Geranyl-geranylation of the small GTPase Rho is catalyzed by geranyl–geranyl transferases, which are inhibited by GGTI-2Z

Small Molecule Modulators of Protein Lipidation

For the above examples, it is clear that lipidation is an essential modification for the activity of many disease-related proteins. For example, src-family kinases (c-Src, Fyn, Lck, Lyk) implicated in a variety of cancers are myristoylated and often palmitoylated. Myristoylated HIV-gag is an attractive target in the treatment of HIV infection. Oncogenic Ras is always prenylated and often palmitoylated. Importantly, some of these lipidated disease targets (e.g., Hh) have no enzyme activity of their own, while the enzymatic activity of others (e.g., Ras) has proven intractable to pharmacological perturbations (Cox et al. 2014). Thus, their lipid modifications, and the enzymes associated with these, may be attractive options for inhibition of pathogenic activity via pharmacological agents (Resh 2012). In the following section, we present a non-exhaustive overview of several exemplary studies of targeting protein lipidations for interference with pathogenic protein function (Fig. 1).

Inhibition of Cholesterol Synthesis by Statins

Statins are a class of drugs that are widely used to lower circulating cholesterol levels via interference with cholesterol biosynthesis. Statin treatment has been shown to affect Hh signaling in vitro (Cooper et al. 2003), with the potential for interfering with development Hh-associated cancers (e.g., basal cell carcinoma) (Tang et al. 2007). This effect is potentially related to the cholesterolylation of these secreted morphogens, although this has not been directly demonstrated.

Separately, although statins are highly effective and relatively specific at inhibiting cholesterol synthesis, pleiotropic effects have been reported. Statins inhibit the rate-limiting enzyme in the mevalonate pathway (3-hydroxy 3-methyl glutaryl CoA reductase), an essential intermediate for cholesterol synthesis, but also for the production of isoprenoids involved in prenylation. It is likely that some of the off-target effects of statins in cultured model systems, and possibly in vivo, are related to statins' interference with protein prenylation [discussed in detail in Gazzerro et al. (2012)]. This effect was clearly demonstrated in *Caenorhabditis elegans*, which lack the branch of the mevalonate pathway leading to cholesterol synthesis, but nevertheless display profound developmental defects upon statin treatment, associated with the unfolded protein response (Morck et al. 2009).

Inhibition of GPI-Anchoring

Arising as a false positive hit in a high-throughput screen for compounds targeting heat shock proteins in *Saccharomyces cerevisiae*, came the discovery of gepinacin, a selective antifungal agent. Making use of the genetic tractability of *S. cerevisiae*, gepinacin was found to target Gwt1, an acyltransferase essential for the biosynthesis of fungal GPI anchors, but not its functional mammalian ortholog PIG-W. The therapeutic implication of this inhibition were revealed in the less genetically tractable but more therapeutically relevant fungus *Candida albicans*: gepinacin impaired fungal filamentation and exposed cell-wall β-glucan, thereby promoting a macrophage-mediated immune response (McLellan et al. 2012).

Inhibitors of Myristoylation

Although several disease associated proteins—including src-family kinases, HIV-gag proteins, and the α subunits of trimeric G-proteins—are myristoylated by *N*-myristoyl transferase isoforms 1 and 2, this modification has not been widely targeted for inhibition because of the hundreds of other myristoylated protein substrates, many of which are essential for cell viability. However, the evolutionary divergence between the human, fungal and parasitic protozoan *N*-methyl transferases (NMT) has been exploited in the development of species-specific inhibitors against *Plasmodium falciparum, Trypanosoma brucei* and *Leishmania donovani* for treating malaria, sleeping sickness (trypanosomiasis), and leishmaniasis, respectively. High-throughput screening identified four series (aminoacylpyrrolidines, piperidinylindoles, thienopyrimidines, and bisphenyl derivatives) of Leishmania-selective inhibitors with considerable selectivity over all other NMTs tested (Hutton et al. 2014; Bell et al. 2012). Co-crystallization with aminoacylpyrrolidine derivatives revealed the active diastereoisomer, facilitating structure-guided fusion with the piperidinylindoles, leading to increased potency.

Another example of targeting myristoylation for therapeutic purposes is by exploiting differences in NMT expression levels. Downregulation of human NMT expression and activity were shown in HIV-1-infected T cell lines. Chronically infected cells with downregulated NMTs showed increased sensitivity to synthetic NMT inhibitors (serinal derivatives) when compared to those with acute HIV infection. Thus, although NMT-targeted inhibitors may affect host cells, there may be a therapeutic window to specifically target HIV-infected cells, making human NMTs a potential target in the treatment of HIV (Takamune et al. 2002).

Prenylation Inhibitors

Prenylation and its associated proteolytic processing are essential for the proper localization and function of many cellular proteins associated with oncogenesis, most notably the small GTPases of the Ras family (H-Ras, N-Ras, and the splice variants K-Ras 4A and B), which act as signal transducers for growth factor receptors at the cell surface. Ras was the first human oncogene discovered and is mutated in a significant number of cancers (Pylayeva-Gupta et al. 2011). Despite being one of the first, and still most sought after targets for anticancer drug discovery, an effective small molecule modulator of Ras activity remains elusive [although interference with downstream effectors, e.g., MAPK, have shown some clinical efficacy (Cox et al. 2014)]. Targeting Ras activity directly is difficult because: (1) the very high (picomolar) affinity for its enzymatic substrate (GTP) prohibits competitive inhibition; (2) allosteric inhibition is challenging due to the absence of well-defined ‘druggable’ pockets. Thus, there has been great interest in pharmacological intervention of Ras processing [reviewed in Gysin et al. (2011)].

Ras interacts with both its activators and downstream effectors on the cytosolic surface of membranes, most often the plasma membrane. Thus, it is no surprise that membrane anchorage is indispensable for Ras activation (von Lintig et al. 2000; Meder and Simons 2005). The mechanisms of anchorage are somewhat isoform specific: all the four Ras isoforms share the CAAX box that specifies prenylation and proteolytical processing, and all require a second signal for stable membrane attachment. For K-Ras, that signal is a polybasic stretch of C-terminal lysine residues; for N- and H-Ras, it is a single or double palmitoylation, respectively (Hancock et al. 1990; Song et al. 2013). Because inhibition of Ras association with the membrane interferes with its mitogenic activity (Cuiffo and Ren 2010), targeting the lipid modifications that mediate this association is an attractive paradigm for inhibiting Ras hyperactivation. This possibility has been explored using prenylation inhibitors. Ras CAAX box peptidomimetics were designed to compete for the enzyme farnesyl transferase (FTase) (Reiss et al. 1990). These peptides were cell-impermeable, so mixed CAAX peptidomimetics with “AA” replaced with benzodiazepine or aminomethylbenzoic acid (C-BZA-M/C-AMBA-M) were designed to increase membrane permeability. These were highly potent ($IC_{50} < 1$ nM) and blocked the attachment of farnesyl to Ras; however, this effect was nonspecific, also affecting the modification of nuclear lamins, among other targets (James et al. 1993). Targeting the prenyl side of the lipidation reaction, farnesyl pyrophosphate (FPP) analogs (Patel et al. 1995), as well as bisubstrate analogs targeting both Ras and FPP have been explored with the aim of achieving target-specific inhibition of FTase (Lannuzel et al. 2003). The most successful have been FTase inhibitors identified by high-throughput screening and optimized by structure-directed medicinal chemistry, e.g., Lonafarnib (SCH66336 IC_{50} 1.9 nM—competitive and 25,000 fold more specific to FTase compared to GGTase inhibition) and Tipifarnib (R115777, IC_{50} 7.9 nM), which have achieved some clinical success and are used in combination antineoplastic chemotherapies (End et al. 2001). Unfortunately, there remain some significant challenges for prenylation inhibitors as anti-oncogenic Ras therapies. FTase and geranyl–geranyl transferase (GGTase) have some target specificity, but when one is inhibited, cross-prenylation can compensate (Fiordalisi et al. 2003). Although combinatorial therapies can simultaneously inhibit both sets of prenylation enzymes—for example, the pyrophosphate analog GGTI-2Z in combination with lovastatin (Sane et al. 2010)—the combined inhibition of both prenyl precursors leads to loss of function of the entire RAS superfamily, along with other targets of prenylation, whose functions are essential for the cell (Gysin et al. 2011; Konstantinopoulos et al. 2007). Thus, while the individual treatments (e.g., FTase inhibitors alone) are not sufficiently effective, the combination therapies are untenably toxic (Lobell et al. 2001). An exciting recent development was the identification and inhibition of the interaction between K-Ras and a protein called PDEδ, which appears to modulate the spatial organization of K-Ras by facilitating its diffusion in the cytoplasm, essentially acting as a K-ras GDI. The identified inhibitors bind to the prenyl binding pocket of PDEδ with nanomolar affinity and thereby inhibit oncogenic signaling and suppress the K-Ras dependent proliferation of human ductal adenocarcinoma cells in vitro and in vivo (Zimmermann et al. 2013).

Hutchinson–Gilford progeria syndrome (HGPS) is a very rare, multisystem disorder, leading to premature aging and death in children. The underlying molecular mechanism is a point mutation in the Lamin-A gene (LMNA), which alters the splicing of the pre-mRNA prelamin A, resulting in a 50 amino acid deletion mutant called progerin. The mutant protein progerin is farnesylated, carboxymethylated, and targeted to the nucleus, where it disturbs nuclear morphology (Yang et al. 2008). Since the farnesyltransferase inhibitor (FTIs) lonafarnib ameliorated disease phenotypes in preclinical studies, it was tested in a clinical trial involving 25 patients who were treated for a minimum of 2 years. Lonafarnib treatment improved the vascular stiffness, bone structure, and audiological status in some of the subjects and improved survival in all of them (Gordon et al. 2012).

Modulators of Palmitoylation

S-palmitoylation of Ras

In contrast to the non-specificity inherent to inhibition of prenylation, selective inhibition of Ras variants may be achieved through targeting their palmitoylation. This selectivity is potentially possible due to the multiplicity of known palmitoyl transferases and characterization of the one associated with Ras palmitoylation, the zDHHC9-GCP16 complex (Greaves and Chamberlain 2011; Swarthout et al. 2005; Mitchell et al. 2006; Salaun et al. 2010). Currently, only a single, nonspecific inhibitor of protein S-palmitoylation is available, the palmitic acid analog 2-bromopalmitate (2-BP) (Webb et al. 2000). This inhibitor can covalently modify the active site of DHHC PATs in vitro (Zheng et al. 2013), thus showing a 'suicide inhibitor' mechanism of action. Moreover, recent demonstrations of promiscuity prevent its clinical utility: the use of click-chemistry enabled 2-BP analogs identified hundreds of covalent targets, including not only the DHHC proteins, but also a broad variety of palmitoylated proteins, as well as some non-palmitoylated proteins (Zheng et al. 2013; Davda et al. 2013). Other compounds with reported inhibitory potential for Ras palmitoylation are 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b]thiophen-3-one, a reversible nonspecific inhibitor of DHHC PATs with potency comparable to that of 2-BP (Jennings et al. 2009), and the less potent antibiotics cerulenin and tunicamycin (Omura 1976; Lawrence et al. 1999; De Vos et al. 2001), all of which are unlikely to be sufficiently specific for clinical utility. Therefore, there remains a dire need and tremendous opportunity for selective, potent inhibitors of palmitoylation that can serve as both molecular tools to probe the physiology of Ras palmitoylation and starting points for anticancer drug discovery.

With the hypothesis that interfering with either direction of the palmitoylation/depalmitoylation process would affect Ras localization and function, a potent inhibitor of the putative depalmitoylating enzyme for H-Ras and N-Ras (APT1) was developed by a knowledge-based bioinformatics strategy called 'protein structure similarity clustering' (PSSC). This approach identified a set of enantiomers Palmostatin A-D as inhibitors of APT1. Palmostatin B exhibited greater affinity in vitro for APT1 and APT2 compared to other lipases tested (PLA1, PLA2, PLD, and PLCβ), and was demonstrated to interfere with the dynamic acylation of Ras (Dekker et al. 2010; Dekker and Hedberg 2011). Since APT1 has a wide range of substrates, structural characteristics of the substrates were used to guide development of more potent derivatives, resulting in Palmostatin M, a nanomolar inhibitor of APT1 (Hedberg et al. 2011). However, inhibitory activity of the Palmostatin analogs against APT1 has yet to be directly demonstrated in cells or in vivo. Other inhibitors for palmitoyl protein thioesterases include the triazole urea derivative ML21, which reacts covalently in vitro with APT1 and 2 (LYPLA1 and 2) (Adibekian et al. 2010). Moreover, in vivo potency against the depalmitoylating enzymes has been demonstrated for reversible piperazine amide derivatives (Adibekian et al. 2012).

Alternative Palmitoylation/Acylation

Dysregulation of Wnt signaling through mutations in downstream signaling components (β-catenin), overexpression of Wnt ligands, or silencing of Wnt inhibitor genes occurs in multiple cancers incl. pancreatic, colon, and hepatocellular carcinoma (Polakis 2012). Wnt proteins are O-palmitoleated by porcupine (Porcn), an MBOAT family acyl transferase that modifies Ser 209 of Wnt 3a. Recently, a high-throughput, cell-based screen for Wnt secretion discovered a specific and potent PORCN inhibitor, LGK947. This compound inhibits Wnt signaling both in vitro and in vivo, and appears to be effective in mouse tumor models (Liu et al. 2013).

An exciting recent development for drug discovery of palmitoylation inhibitors comes from the discovery of a specific inhibitor of Shh palmitoylation. Using a target-based high-throughput screen with a Shh mimetic peptide, 4 unique compounds were selected that displayed low micromolar inhibitory activity against the Shh palmitoylation enzyme Hhat. These compounds effectively blocked downstream signaling in cell lines, suggesting potential utility for Hh associated chemotherapy (Petrova et al. 2013).

Finally, ghrelin is a growth hormone secretogogue and orexigenic (appetite stimulating) peptide that binds its receptor (GHS-R1a) when it is modified by an 8-carbon fatty acid, i.e., octanoylated. This modification is mediated by the unique enzyme ghrelin *O*-acetyl transferase (GOAT), which has been identified as a promising therapeutic target for obesity and other metabolic disorders driven by impaired glucose homeostasis. Recently a bi-substrate analog GO-CoA-Tat peptide antagonist was developed to target the ternary complex between GOAT, octanoyl CoA and ghrelin. This tripartite macromolecule comprises the minimal amino acid sequence of the ghrelin peptide required for recognition by GOAT and an amino hexanoyl linker to an eleven amino acid sequence derived from HIV-tat at the C-terminus added to enhance its cell permeability. The compound inhibited the production of acylated ghrelin with an IC_{50} of 5 μM in vitro, also exhibiting selectivity for GOAT over other acyl transferases tested. Treatment with GO-CoA-Tat reduced the serum levels of acyl-ghrelin, reduced the weight gain in mice fed on a high fat diet (Barnett et al. 2010), and decreased plasma levels of aldosterone and corticosterone in rats (Rucinski et al. 2012), suggesting a bright future for this unique therapeutic.

Conclusions and Perspectives

Because protein lipid modifications are widespread and often indispensable for protein localization and function, they potentially represent attractive targets for pharmacological intervention. This review discusses several examples in which targeting protein lipidation has been used as a viable strategy to obtain therapeutics of clinical value. However, several issues limit the utility of targeting protein lipidation. These include the lack of suitable, well-characterized high-throughput screening assays that target the lipid-modifying enzymes or the lipidation process. Since many of the lipid-modifying enzymes are transmembrane proteins, they are difficult to crystallize and study in molecular detail, posing a further challenge to structure-guided drug discovery (Chavda et al. 2014). This fact is evidenced by the observation that most successes described in this review were results of screens of diverse compound libraries, followed by structure-guided medicinal chemistry refinement, including the GPI anchor inhibitor gepinacin, myristoylation inhibitors, and prenylation inhibitors (e.g., lonafarnib). An example of a structure-guided success story are the palmostatins that inhibit Ras depalmitoylation.

Other challenges are that most lipid modifications are irreversible, and the modifying enzymes have a broad range of substrates. For example, humans have only three enzymes for prenylation, and only two for myristoylation, to serve hundreds of targets. Moreover, these enzymes are redundant in function, with inhibition of one evoking the activity of another (albeit with lower affinity for the target). An example is the failure of FTIs in inhibiting Ras, which were alternatively geranyl geranylated. A widely explored paradigm to achieve substrate specificity when targeting enzymes that modify many substrates, is the development of bi-substrate analogs that incorporate the structural features of the modified protein in the design of inhibitors targeting the modifying enzyme. This configuration is designed to target the enzyme-substrate intermediary complex, rather than the enzyme or the substrate itself. Examples for this strategy include inhibitors developed against FTase, GGTase, as well as GOAT. However, appending peptides to the small molecules compromises their pharmacokinetics. Successful small molecules in such cases have also been developed by exploiting the evolutionary divergence of lipidation enzymes between humans and pathogens. Examples are myristoylation inhibitors for Leishmaniasis and antifungals targeting GPI anchor synthesis.

Palmitoylation is a widely occurring protein modification, affecting hundreds if not thousands of mammalian proteins. Palmitoylation is unique among the lipid modifications in that it is mediated by a large number of enzymes, suggesting the possibility of specific perturbation of palmitoylated targets. It is also the only reversible modification, providing another point of vulnerability for pharmacological interference. Essential for development of specific pharmacological targeting are profiles of the palmitoylated proteome and enzyme-substrate specificities—efforts to characterize these are underway, but are far from complete. Thus far, the palmitoylation machinery appears largely redundant, both in terms of many enzymes modifying a specific substrate, and many targets for any given enzyme. This situation would not be ideal for drug development, as off-target side effects and lack of efficacy may result. Here, target-based/process-based approaches may likely yield specific hits through well-designed high-throughput screening and counter-screening strategies.

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