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Aβ-degrading proteases:therapeutic potential in Alzheimer disease

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Abstract

The amyloid ß-protein (Aß) is well established to play an indispensible role in the pathogenesis of Alzheimer disease (AD). Aβ is subject to proteolytic degradation by a diverse array of peptidases and proteinases, known collectively as Aβ-degrading proteases (AβDPs). A growing number of AβDPs have been identified, which impact Aß powerfully and in a surprising variety of ways. As such, AßDPs hold considerable therapeutic potential for the treatment and/or prevention of AD. Here we critically review the relative merits of therapeutic strategies targeting AßDPs as compared to current Aß-lowering strategies focused on immunotherapies and pharmacological modulation of Aß-producing enzymes. Several innovative advances have increased considerably the feasibility of delivering AßDPs to the brain or enhancing their activity in a non-invasive manner. We argue that therapies targeting AßDPs offer numerous potential advantages, which should be explored through continued research into this promising field.

Introduction

Alzheimer disease (AD) is a debilitating and increasingly common neurodegenerative disorder that results in progressive loss of memory, cognition, language skills, and personality traits [1]. Histopathologically, the disease is characterized by progressive accumulation of “plaques” composed of the amyloid-ß protein (Aß) and “tangles” comprised of hyperphosphorylated forms of the microtubule-associated protein, tau [1]. Currently, over 35 million people worldwide are afflicted [2], including 5.3 million individuals in the USA [3]. As people are living longer and the leading edge of the baby-boomer generation ages, AD is growing to epidemic proportions. Recent studies indicate that AD affects a larger portion of the population than previously believed, now ranking as the 3rd leading cause of death, exceeded only by heart disease and cancer [4]. The financial burden imposed by the marked increase in AD cases, as well as the stress on caregivers and family members, represents an unprecedented global public health challenge. Alzheimer’s Disease International (ADI) estimates a global annual cost of dementia, of which AD is the leading cause, of ~$818 billion or 1% of global GDP [2]. To date, over 100 AD clinical trials

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Compliance with Ethical Standards

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have been conducted [5] and—sadly—none has proven effective at halting or even slowing disease progression significantly. There is, therefore, a vital need to investigate alternative treatment strategies that can prevent or treat this debilitating disease.

Centrality of Aß mismetabolism

Advances in human molecular genetics have contributed inestimably to our understanding of the molecular pathogenesis of AD, demonstrating that specific perturbations to Aß metabolism are sufficient to trigger the full spectrum of the disease [6]. However, the cause of AD is known with certainty in only a tiny minority (~1%) of all patients, specifically early-onset, familial AD (FAD) cases attributable to dominant-negative mutations in 3 genes: the amyloid precursor protein (APP) and presenilin 1 and 2 (*APP*, *PSEN1*, *PSEN2*) [7]. Several hundred FAD-linked mutations have been identified, and all universally increase either total Aß levels or specifically the relative abundance of longer, more pathogenic forms of Aß such as Aß42 [8]. Individuals with Down syndrome (trisomy 21), who carry 3 copies of the *APP* gene on chromosome 21 and therefore produce 50% more Aß than normal, also invariably develop AD-type pathology [8]. Collectively, these genetic findings provide exceptionally strong evidence that perturbed Aß metabolism plays a causal role in triggering the full spectrum of AD-type pathology, including those elements (e.g., NFTs and neurodegeneration) that only appear at later stages in the disease [7].

For the vast majority of AD cases, however—commonly referred to as sporadic, idiopathic or late-onset AD—Aß accumulates abnormally, but the operative mechanisms remain less certain. Increases in Aß production not attributable to FAD-linked mutations have been documented to occur in some AD patients [9, 10]). However, it has been hypothesized that reduced *clearance* of Aß—either alternatively or in addition to increased Aß production—may be operative in the etiology of many cases of sporadic AD [6, 11]. Consistent with this idea, allelic variations in apolipoprotein E (ApoE), which is implicated in the clearance of Aß, dramatically modulate AD risk [12].

The hypothesis that impaired Aß clearance *per se* is occurring in some sporadic AD cases remained largely speculative for many decades. In recent years, however, powerful techniques have been developed that, through the transient administration of radiolabeled amino acids to human subjects, permit the accurate quantification of the rates of Aß production and clearance in the cerebrospinal fluid (CSF) of living patients [13]. These elegant studies have confirmed that Aß clearance—or more accurately, the rate of Aß clearance relative to that of production—is indeed decreased in sporadic AD cases [14].

Lessons from therapies targeting Aß production

Given the certitude that excessive production of Aß causes familial forms of AD, it is unsurprising that most Aß-lowering therapeutics strategies under development target the enzymes that mediate Aß production (or, as considered in a subsequent section, Aß itself). Aß is generated from APP, a type 1 membrane protein, via the action of proteolytic enzymes known as “secretases” because their action results in the secretion of Aß and/or the large extracellular ectodomain of APP [15]. In the processing pathway that results in Aß production, APP is initially cleaved by ß-secretase, an aspartic protease known as

beta-site APP-cleaving enzyme 1 (BACE1) [15], releasing a fragment known as sAPPß. The remaining membrane-bound C-terminal fragment (termed C99) is subsequently cleaved by γ-secretase, an intramembraneous complex of four proteins, the active-site of which is formed by presenilin-1 or −2 [15].

Two aspects of γ-secretase are noteworthy: first, it cuts at positions within the membrane, an unusual site for hydrolysis to take place. Second, unlike most proteases, γ-secretase does not cut a specific peptide bond, but instead cleaves at any of several sites, resulting in the generation of a mixture of Aß species from 37 to 48 amino acids in length, with notable examples being Aß40 and Aß42 [15]. FAD-linked mutations in presenilins cause the production of Aß42 relative to Aß40 (the Aß42/40 ratio) to increase [16], and this shift that has been strongly implicated in the pathogenesis of FAD [17, 18].

Once the mechanistic details of Aß processing were elucidated, it became clear that there were two fundamental ways to reduce Aß production: via inhibition of BACE1 or the presenilin/γ-secretase complex. Outright inhibition of these proteolytic activities, however, has proved to be problematic, primarily because they process numerous substrates besides APP. For example, although genetic deletion of BACE1 in mice results in viable animals that exhibit absolute cessation of Aß production [19, 20], several more subtle, but worrisome phenotypes, such as hypomyelination, were revealed upon closer analysis [21]. More recently, ß-secretase has been shown to be an active sheddase in mouse primary neurons, where it contributes to the secretion of approximately one fifth of identified shed proteins [22], including neuregulin, which has important functions in myelination [23]. Furthermore, chronic pharmacological inhibition of BACE1 has resulted in adverse effects in mice [24]. Finally, potential secondary effects have been observed in clinical trials of BACE1 inhibitors [25].

Pharmacologic inhibition of presenilin/γ-secretase has proved to be problematic, as well, for similar reasons. In mice, genetic deletion of presenilin-1 results in premature lethality [26], attributable in part to impairments the processing of notch1, an intramembrane protein involved in cell fate determination during development [27]. As was true for BACE1, numerous substrates for presenilin/γ-secretase besides APP and notch1 have been identified over the years [28], raising further questions about the viability of outright inhibition of γ-secretase. Given this, it is perhaps unsurprising that the γ-secretase inhibitor (GSI) semagacestat [29], although effective at lowering Aß in humans [30], actually resulted in cognitive worsening when tested in long-term clinical trials [31, 29].

Notwithstanding the theoretical and empirical problems with GSIs, there is considerable hope for a class of compounds known as γ-secretase modulators (GSMs). Due to the complexity and unusual structure of the presenilin/γ-secretase complex [32], compounds have been identified that modulate, rather than block, γ-secretase activity in both substrate-specific and cleavage site-specific ways [33]. Thus, it has been possible to develop “notch-sparing” inhibitors of APP processing [33], as well as compounds that specifically reduce the Aß42/40 ratio [34]. Although GSMs hold perhaps the greatest promise among those therapies targeting Aß production, there is some concern about focusing too narrowly on Aß- and notch-specific effects, to the exclusion of possible influences on the many

other substrates processed by presenilin/γ-secretase. This caveat is especially relevant given accumulating evidence that the pathogenicity of certain FAD presenilin mutations may result from an overall decrease in γ-secretase function in addition to their effect on the Aß42/40 ratio [35].

Lessons from Aß immunotherapies

The other approach to lowering Aß that has been extensively explored is that of enhancing clearance of cerebral Aß through active or passive immunization with antibodies against Aß. Like therapies targeting AßDPs, discussed below, this approach has the advantage of circumventing the deleterious effects associated with ß- and γ-secretase inhibition. Active immunization proved highly effective in removing brain Aß in mouse models [36–40] as well as humans [41], and early studies showed some evidence of blunting cognitive decline in some AD patients [42]. However, this approach also triggered deleterious immune-mediated reactions in a subset of patients [43]. To overcome these problems, considerable effort has explored the therapeutic potential of passive immunization, wherein the patient is administered humanized monoclonal antibodies against Aß. Unlike active immunization, this approach has the advantage of being reversible and also avoiding the deleterious consequences of cell-mediated immunity [44]. Nevertheless, despite a number of ambitious clinical trials, none have proven unambiguously to blunt cognitive decline. Moreover, concerns have been raised about the ability of Aß antibodies to translocate Aß from the parenchyma to the brain vasculature, together with concerns about pro-inflammatory responses triggered by the Fc portion of anti-Aß antibodies [45]. Thus, despite the seemingly great promise of Aß immunotherapy, sufficient uncertainty about the efficacy and safety of this approach remains to warrant continued exploration of alternative therapeutic strategies.

General features of AßDPs

Given the lack of success obtained with Aß immunotherapies and drugs targeting Aß production, it is reasonable to consider whether therapies targeting AßDPs might prove to be viable—or even superior—as alternatives for lowering of brain Aß. As a general class, it is difficult to overstate the importance of AßDPs for the regulation of cerebral Aß levels. It has been estimated that over 8 decades of life the average individual generates >10 kg of Aß [11]; since only a tiny fraction of Aß incorporates into insoluble aggregates such as plaques or cerebral amyloid, it follows that the overwhelming majority of Aß peptides produced are ultimately destroyed by AßDPs. In a particularly striking illustration, inhibition of just a subset of just one class of proteases (zinc-metalloproteases) via i.c.v. administration of phosphoramidon produced a >400% increase in cerebral Aß within 30 min [46, 47]. When compared, for example, to trisomy 21, wherein a mere 50% increase in Aß gives rise to AD-type pathology by the 4th decade of life, it is easy to see how even a partial impairment in Aß degradation could play a significant role in AD pathogenesis.

It is important to emphasize that proteolytic degradation of Aß represents just one of several mechanisms of Aß clearance that operate in parallel. Cerebral Aß levels are also regulated by such processes as cell-mediated clearance and active and passive transport of Aß across the blood-brain barrier [48]. It is difficult to rank the relative importance of these different clearance mechanisms, in no small part because they ultimately culminate in proteolytic

degradation (e.g., via lysosomal proteases). From a therapeutic perspective, however, these highly complex pathways may be more difficult to manipulate than AßDPs, which represent discrete therapeutic targets.

AßDPs can be sorted into several broad functional categories (Table 1). *Endogenous regulators* of Aß are AßDPs involved in the regulation of Aß under physiological conditions. Examples include insulin-degrading enzyme (IDE), neprilysin, and endothelin-converting enzymes-1 and −2 (ECE1; ECE2) [49]. By definition, deletion or inhibition of an endogenous regulator will necessarily produce elevations in cerebral Aß in the absence of pathology. It is notable that a large number of AßDPs fit this category, because this demonstrates that Aß clearance is operating *at capacity*, such that the elimination of any of several AßDPs will increase cerebral Aß. By the same token, increasing the activity or levels of *any* AßDP—including one not normally involved in Aß catabolism—will have the effect of lowering Aß levels; a finding that has been demonstrated multiple times in mouse models.

The second functional category is *pathological regulators*, which are AßDPs that are only active in the presence of overt Aß pathology. Plasmin is prime example of a pathological regulator [49]: in the absence of pathology, deletion of the precursor protein for plasmin (plasminogen) gives rise to no net increase in Aß; however, in the presence of aggregated forms of Aß, plasminogen get converted to active plasmin by tissue-type plasminogen activator (tPA), a protease that is sensitive to the presence of beta-pleated sheets.

A final category might be termed *therapeutic regulators*. Although any of the above proteases could be used therapeutically, it is critical to recognize that any protease capable of degrading Aß—even an engineered protease or one not normally expressed in brain—could in principle be used therapeutically [50]. Thus, as compared to the mere two proteases involved in Aß production, it is apparent that AßDPs represent and enormously diverse collection of possible therapeutic targets, which have only barely been explored for their therapeutic potential.

AßDPs can be further distinguished in terms of their proteolytic properties and the substrates they can act on. As is true for all proteases, AßDPs can include endoproteinases, aminopeptidases and carboxypeptidases. The latter class is of special interest, since certain proteases—such as cathepsin B—can convert the longer, more pathogenic forms of Aß, such as Aß42, to shorter species, such as Aß40 or Aß38, via carboxypeptidase activity [51]. Finally, given that Aß can exist in monomeric forms as well as multiple distinct aggregated forms [52, 53], ranging from dimers and trimers, to a hypothesized dodecameric species termed Aß*56, to protofibrils, fibrils, and, finally, as macroscopic “plaques,” AßDPs can be categorized in terms of which Aß species they are capable of hydrolyzing (Table 1). For example, pure peptidases, such as IDE, can act only on monomeric Aß species, while other AßDPs can degrade oligomeric or fibrillar forms of Aß [50].

The question of which species/conformations of Aß different AßDPs target may be of particular therapeutic relevance. For example, high-level transgenic overexpression of neprilysin in a mouse model of AD has been shown to prevent the formation of extracellular amyloid deposits completely [54]. Nevertheless, oligomeric Aß—a particularly neurotoxic

form of Aß [52]—was found to be unchanged by neprilysin overexpression in these same animals; moreover, the mice lacking plaques (but containing oligomers) also showed cognitive defects identical to APP transgenic mice not overexpressing neprilysin [55]. In a related vein, by virtue of their particular subcellular localization profiles and pH optima, different AßDPs can target spatially distinct "pools" of Aß (e.g., extracellular, endosomal, lysosomal) (Table 1) [56]. As is true for the various species of Aß, some pools of Aß are more strongly implicated than others in AD pathogenesis [57, 58]. Intriguingly, there is some evidence that extracellular pools of Aß may not be the most relevant to cognitive defects. For instance, overexpression of Aß42 exclusively in the extracellular space (using BRI-Aß42 fusion proteins [59]) led to the development of robust amyloid plaques [60], but failed to affect cognitive performance [61]. These intriguing findings could explain why therapies that target extracellular Aß, such as immunotherapies—have failed to impact cognitive decline.

Several additional intrinsic features of AßDPs render them particularly attractive as therapeutic targets. First and foremost, the general strategy of targeting the removal of Aß after it is produced obviates all of the aforementioned concerns about the machinery involved in Aß production. Moreover, relative to other approaches, such as immunotherapies or inhibitors of Aß aggregation, which necessarily act stoichiometrically, AßDPs have the distinct advantage of acting *catalytically* and *irreversibly* to remove Aß [62]. Thus, a very large reduction in Aß levels can be effected by a relatively small increase in the level or activity of a specific AßDP [62]. Finally, whereas Aß production necessarily occurs exclusively in the proximity of ß- and γ-secretase, Aß degradation can take place any arbitrary distance from the sites of Aß production [56]. Indeed, some reports have suggested that increasing Aß degradation (or sequestration [63]) in the periphery can lower cerebral Aß levels [64, 65], although these initial results were not confirmed in several well designed studies [66, 67]. Nevertheless, from a drug development perspective, the fact that AßDP-targeting therapies can operate at some distance from the source of Aß production represents a distinct advantage over existing Aß-lowering strategies.

Because AßDPs degrade a number of substrates besides Aß, it is important to consider the potential off-target effects of increasing particular AßDPs. Although this is a valid concern, it should be noted that AßDPs are in no way unique in this regard; the secretases involved in Aß production also degrade numerous substrates besides APP, yet this has not prevented secretase inhibitors from being intensively investigated in clinical trials. Moreover, as is true for γ-secretase, there is strong evidence that certain AßDPs can be modulated by small molecules in a highly substrate-specific manner [68, 69]. Furthermore, if certain forms of AD are attributable to deficiencies in specific AßDPs, restoring the activity of that AßDP to normal levels would not be expected to have side effects. Finally, animal modeling studies involving high-levels overexpression of NEP or IDE have not revealed overtly deleterious consequences [54, 70, 71, 55]. Nevertheless, the potential for off-target effects is an important one, and it will therefore be of critical importance to comprehensively evaluate the potential side effects of candidate therapeutic AßDPs in animal studies prior to any clinical applications.

Therapeutic approaches to targeting AßDPs

At first glance, AßDPs would appear to be most amenable to gene therapeutic approaches. Animal modeling studies have confirmed the viability of this approach. Long-term viral-mediated expression of neprilysin, for example, has been shown to lower Aß levels and ameliorate Aß-associated cognitive deficits [71]. However, there remain serious theoretical and practical concerns about the clinical use of potentially risky viral vectors and associated invasive procedures to deliver them. Nevertheless, gene therapies are already being tested in AD patients. For instance, a clinical trial of nerve growth factor gene delivery was conducted in 2001 [72], and post-mortem analysis revealed significant effects lasting as long as 10 years [73]. It is notable, however, that two of the patients in this trial were severely affected by the intrusive surgical procedure, one of whom died [72]. Numerous advances have been made in the intervening years, however, that suggest less-invasive means of gene-delivery might be feasible [74]. For example, viral delivery of neprilysin in rats has been shown to be enhanced significantly using convention-enhanced delivery [75]. A modified adeno-associated virus (AAV) vector has been developed by Saido and colleagues that produced neuronal expression of neprilysin throughout the brain of mice following peripheral delivery [76].

Delivery of AßDPs to the CNS does not necessarily require the administration of viral vectors or genetically modified cells. For example, Masliah and colleagues developed a recombinant form of neprilysin [76] containing a brain-transport peptide [77]. In initial studies, this fusion protein was delivered via a lentiviral vector administered peripherally and expressed primarily in liver and spleen [76]. Using this approach, the recombinant neprilysin was shown to effectively lower cerebral Aß levels, plaque burden and also reverse cognitive deficits. Notably, in subsequent studies this team succeeded in obtaining similarly positive results via peripheral administration of the recombinant fusion protein alone [78]. Thus, with sufficient improvements, it may be feasible to deliver AßDPs intravenously, in similar manner to Aß immunotherapies.

Can small molecules be developed that increase the activity of AßDPs? Generally speaking, it is widely assumed that it is not feasible to increase the activity of a protease, but this is not, in fact, true. For example, many proteases are regulated by endogenous inhibitors, and it is possible to develop compounds that block the interaction of the protease and its inhibitor, thereby disinhibiting the protease. For example, Wyeth developed a small-molecule activator of plasmin which showed efficacy in an animal model of AD [79]. This compound acted by displacing plasminogen activator inhibitor-1 (PAI1), an endogenous inhibitor of tissue-type plasminogen activator (tPA), which converts plasminogen to plasmin [79]. Since, as mentioned above, tPA is activated by beta-pleated sheets, including those formed by Aß [80], this compound effectively lowered the threshold for tPA-mediated plasmin activation by Aß. Direct activators of other enzymes [81], including AßDPs such as IDE [68, 82], have also been identified. Thus, the “druggability” of AßDPs is greater than has been widely assumed.

Rather than pharmacologically activating AßDPs, it is also possible to increase their expression. For example, levels of just a single AßDP, neprilysin, have been shown to increase in response to a wide variety of factors, including substance P [83], Aß [84, 85], to

the intracellular domain of APP [86, 87], estrogen [88], and even environmental enrichment [89]. Saido and colleagues also found that administration of somatostatin reduced brain Aß levels via activation of neprilysin, and suggested that drugs targeting somatostatin receptors might be candidates as Aß-lowering targets [90]. Finally, given the great advantages of natural products in terms of safety and availability, it is notable that green tea extract has been shown to increase neprilysin levels [91]. Other dietary supplements have been shown to reduce Aß in animals models [92] and should be investigated for possible effects on AßDPs.

As is true for all Aß-lowering therapeutics, the question of when to initiate therapies targeting AßDPs remains critical. Basic research has not yet addressed this question directly, but the lack of success of clinical trials with other Aß-lowering approaches initiated in patients already showing memory loss suggests that it may be critical to intervene as early as possible in the course of the disease. Given the long-term nature of AD pathogenesis, manipulations that are comparably long-lasting, such as gene-therapeutic approaches, may therefore be particularly advantageous relative to most other, short-acting Aß-lowering therapeutics.

Conclusions

As a general class, AßDPs represent attractive targets for drug development. By virtue of their catalytic nature, AßDPs represent powerful regulators of Aß levels, and their sheer number and variety increases the chances that at least one will be found that can be targeted pharmacologically or expressed or administered therapeutically. Relative to the molecular machinery involved in Aß production, which has been the focus intensive research in both academia and industry, significantly less is known about AßDPs generally, including the complete set of AßDPs, and there have been no efforts to evaluate the risk-reward profiles of different AßDPs in a systematic fashion. Given the many novel aspects of AßDPs considered here, continued research into the possible therapeutic utility of AßDPs is clearly warranted.

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Bullet point summary

- Proteases that degrade the amyloid ß-protein (Aß), which is central to the pathogenesis Alzheimer disease (AD), are powerful regulators of cerebral Aß levels and downstream cytopathological and cognitive sequelae.
- By virtue of their sheer number and diversity, Aß-degrading proteases (AßDPs) offer a rich source of therapeutic targets to combat AD, with several intrinsic advantages over existing strategies to reducing cerebral Aß.
- Recent advances have shown that the activity and/or levels of AßDPs can be enhanced through a variety of non-invasive therapeutic approaches.

Table 1.

Properties of known Aß-degrading proteases

Type	Protease	Substrates[a]		Functional Category[b]	Subcellular localization[c]
		Oligos	Fibrils		
Metallo	NEP	synth	no	E,P,T	ex, ER, G
	NEP2			E	ex, ER, G
	hMMEL				ex, ER, G
	ECE1			E	ex, ER, G, endo
	ECE2			E	ex, ER, G, endo
	ACE				ex, ER, G
	MMP2		yes		ex, ER, G
	MMP9		yes		ex, ER, G
	CD147/EMMPRIN				ex, ER, G, endo
	IDE	no	no	E,T	ex, ER, endo, lyso, mito
	PreP				mito
Serine	Plasmin/tPA/uPA	natural	yes	P,T	ex, ER, G
	Acylpeptide hydrolase				cyto
	Myelin basic protein				ex, ER, G
Aspartate	Cathepsin D		yes	E	endo, lyso
	BACE1			T	endo, lyso
	BACE2		No	E	endo, lyso
Cysteine	Cathepsin B			E,T	ex, endo, lyso
Threonine	Proteasome				cyto

[a]Where known, indicates whether the corresponding AßDP degrades oligomeric (ologos) or fibrillar (fibrils) Aß.

[b]Where known, indicates functional category of each AßDP (E, endogenous regulator; P, pathologic regulator; T, therapeutic regulator).

[c]Indicates subcellular localization (cyto, cytosol; endo, endosomes; ER, endoplasmic reticulum; ex, extracellular space; G, Golgi network; lyso, lysosomes; mito, mitochondria). ACE, angiotensin-converting enzyme; BACE1,2, beta-site APP-cleaving enzyme 1, 2; ECE1,2, endothelin-converting enzyme 1,2; EMMPRIN, extracellular matrix metalloproteinase inducer; hMMEL, human membrane metallo-endopeptidase-like protein; IDE, insulin-degrading enzyme; MMP2,9, matrix-metalloproteinase 2,9; NEP, neprilysin; NEP2 neprilysin 2; PreP, presequence peptidase; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator. For primary references, see ref. [50].